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Doxorubicin induces drug efflux pumps in *Candida albicans*

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Candida albicans is one of the most important opportunistic fungal pathogens. It can cause serious fungal diseases in immunocompromised patients, including those with cancer. Treatment failures due to the emergence of drug-resistant *C. albicans* strains have become a serious clinical problem. Resistance incidents were often mediated by fungal efflux pumps which are closely related to the human ABC transporter P-glycoprotein (P-gp). P-gp is often overexpressed in cancer cells and confers resistance to many cytotoxic drugs. We examined whether cytotoxic drugs commonly used for cancer treatment (doxorubicin and cyclophosphamide) could alter the expression of genes responsible for the development of fluconazole resistance in *Candida* cells in the way they can influence homologous genes in cancer cell lines. ABC transporters (*CDR1* and *CDR2*) and other resistance genes (*MDR1* and *ERG11)* were tested by real-time PCR for their expression in *C. albicans* cells at the mRNA level after induction by antineoplastic drugs. The results were confirmed by a lacZ gene reporter system and verified at the protein level using GFP and immunoblotting. We showed that doxorubicin is a potent inducer of *CDR1/CDR2* expression in *C. albicans* at both the mRNA and protein level and thus causes an increase in fluconazole MIC values. However, cyclophosphamide, which is not a substrate of human P-gp, did not induce ABC transporter expression in *C. albicans*. Neither doxorubicin nor cyclophosphamide could influence the expression of the other resistance genes (*MDR1* and *ERG11)*. The induction of *CDR1/CDR2* by doxorubicin in *C. albicans* and the resulting alteration of antifungal susceptibility might be of clinical relevance for the antifungal treatment of *Candida* infections occurring after anticancer chemotherapy with doxorubicin.

Keywords *Candida albicans*, azoles, ABC transporter, resistance mechanisms, doxorubicin

Introduction

Candida spp. infections have increased in incidence among immunocompromised patients in recent years and have now become the fourth most common bloodstream infections [1–3]. These pathogens pose a serious threat to chemotherapy patients whose immune system is compromised by leukemia or other types of cancer [4,5]. Some molecular processes are linked to the emergence of intractable fluconazole-resistant *Candida albicans* infections [6,7], the most important of which are; (i) upregulation of *CDR1* and *CDR2*, genes encoding multidrug efflux transporters of the ATP-binding cassette (ABC) transporter family, (ii) upregulation of *MDR1*, a major facilitator transporter gene, and (iii) transcription increase of *ERG11*, a gene coding for the drug target enzyme sterol 14α-demethylase or *ERG11* point mutations. The transporter proteins increase active efflux of antifungal agents, and upregulation of *ERG11* raises the amount of the target enzyme, making the intracellular azole concentration insufficient to inhibit the enzyme activity [7–9].

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The therapy of cancer includes several procedures such as surgery, radiotherapy and chemotherapy. Multidrug resistance (MDR) of neoplastic tissues is a persistent problem in cancer chemotherapy [10]. The main cause of MDR is overexpression of P-glycoprotein (P-gp), a member of the human ABC transporter family. This transporter family has broad substrate specificity for several substances, including anticancer drugs, linear and cyclic peptides, HIV protease inhibitors, and several other molecules [11,12]. The putative physiological function of P-gp is to protect cells from toxins and xenobiotics. Substrates of these efflux pumps are cytotoxic compounds of natural origin such as anthracyclines (doxorubicin, daunorubicin), vinca alkaloids (vincristine, vinblastine), epipodophyllotoxins (etoposide, teniposide), taxane, and amsacrine. Doxorubicin, daunomycin, vincristine and mitoxantrone were shown to induce P-gp mRNA in rat, mouse and human cell lines [13,14].

The fungal *CDR* efflux pumps are functionally and structurally related to the human P-gp responsible for MDR. The aim of this study was to investigate whether antineoplastic drugs used in cancer therapy could affect the expression levels of genes responsible for antifungal drug resistance. We tested two commonly used cytostatic drugs, i.e., doxorubicin (intercalating agent and substrate of human P-gp) and cyclophosphamide (alkylating agent and not a substrate of human P-gp). Using a real-time PCR system developed in our laboratory, ABC transporters (*CDR1* and *CDR2*) and other resistance genes like *MDR1* and *ERG11* were tested for their expression in *C. albicans* cells at the mRNA level after induction by the above-mentioned drugs [15]. All PCR results were obtained with three pairs of clinical *C. albicans* isolates from three individuals with HIV infection. Each pair of isolates consisted of an azole-sensitive (pretreatment) isolate and a second isolate that had acquired resistance during fluconazole treatment for recurrent oropharyngeal candidosis (OPC). In addition, a *TAC1* knock-out mutant and its paternal strain were tested by similar approaches. *TAC1* (transcriptional activator of *CDR* genes) is essential for controlling expression of *CDR1* and *CDR2* [16]. The PCR results were confirmed by a β-galactosidase (lacZ) gene reporter system and verified at the protein level by GFP fusion proteins and Western blot analysis as previously described [16,17].

Materials and methods

Strains and culture conditions

C. albicans isolates used for gene expression analyses are listed in Table 1. The isolates were obtained from three AIDS patients by oral washings with sterile 0.9% NaCl solution during recurrent episodes of OPC before and after fluconazole treatment. Fluconazole-susceptible isolates (F1, B1, and Gu1) were recovered before initiating fluconazole treatment. The second set of isolates (F5, B5, Gu5) was recovered from the same three individuals during fluconazole-refractory episodes (no response to 400 mg/day fluconazole) later in the course of HIV infection. The expression levels of *CDR* genes, *MDR1* and *ERG11* genes in these isolates, as well as the MIC values have been described in previously published studies [9,18]. *C. albicans* isolates were classified as susceptible to fluconazole with MICs of $\leq$ 8.0 μg/ml, dose-dependently susceptible with MICs of $>$ 8.0 to $\leq$ 32 μg/ml, and resistant within MICs of $>$ 32 μg/ml [19].

The *C. albicans tac1Δ/Δ* mutant and its paternal strain CAF2-1 have been described in a previous publication [16]. The isolates were kept as frozen stocks at −80°C and subcultured on YPD agar medium in plates (10 g/l yeast extract, 20 g/l peptone, 20 g/l glucose, 15 g/l agar) at 30°C.

RNA extraction and cDNA synthesis including DNase digest

Before RNA extraction, the *C. albicans* isolates were cultured in YPD broth overnight at 37°C. Portions of growth from the overnight culture were used to inoculate fresh YPD broth, and cells were allowed to grow for another 2 h until mid-log phase. Cytotoxic drugs were added in defined (therapeutic) concentrations (20 μg/ml of doxorubicin, 20 μg/ml of cyclophosphamide) to samples designated for treatment and incubated either for 90 min or 24 h. Each strain was concomitantly cultured at both time points without the drug as a control. Fluconazole was not added to the cultures.

RNA was isolated using RNA-PLUS™ (Total RNA Extraction Solution, Qbiogene) according to the manufacturer's guidelines. Before starting the RNA isolation protocol, the fungal cell walls were mechanically disrupted using Lysing Matrix C tubes and the FastPrep® FP120A instrument (Qbiogene). The RNA concentrations were determined by photometry. One microgram of RNA in a 19 μl volume was used for cDNA synthesis. After adding 1 μg of oligo(dT) primer (Invitrogen, Karlsruhe, Germany), the samples were incubated for 10 min at 75°C. The following components were added to each sample: 8 μl of 5 × first strand buffer, 4 μl of 0.1 M dithiothreitol (both Invitrogen), 4 μl of NTPs (10 mM each) (GeneAmp®, Applied Biosystems, Darmstadt, Germany), 20 U of RNasin® Plus Rnase Inhibitor (Promega, Mannheim, Germany) and 1 μl of DNase (Roche, Mannheim, Germany) to eliminate possible DNA contamination. The mixture was incubated at 37°C for 30 min and at 75°C for 5 min to inactivate the DNase. For the final reaction, 20 U of RNasin® Plus RNase Inhibitor (Promega) and 200 U of SuperScript™

Table 1 Strains and clinical isolates of *Candida albicans* used in this study.

Isolates		Date of isolation (mo/day/yr)	MIC_{Flu} (μg/ml)	Description	Reference
Patient 1	F1	07/05/90	3.12	–	[9]
	F5	01/14/91	50	Enhanced *MDR1* and *ERG11* mRNA levels	
Patient 2	Gu1	01/24/90	0.78	–	[18]
	Gu5	11/02/95	> 100	Enhanced *CDR1*, *CDR2* mRNA levels	
Patient 3	B1	09/27/93	0.39	–	[18]
	B5	11/17/94	50	Enhanced *MDR1* mRNA level	
Strains		**Parent strain**	**MICFlu (μg/ml)**	**Description**	**Reference**
CAF2-1		SC5314	≤ 0.125	Parent strain of *TAC1* null mutant	[35]
*tac1*Δ/Δ		DSY2883	≤ 0.125	*TAC1* null mutant	[16]
MDR1-lacZ		DSY449	≤ 0.125	*MDR1* promotor fusion with *lacZ*	[36]
CDR2-lacZ		DSY449	≤ 0.125	*CDR2* promotor fusion with *lacZ*	[36]
DSY3040		DSY294	0.25	Parental strain of Cdr1p- and Cdr2p-GFP strains (azole susceptible)	[22]
DSY3041		DSY296	64	Parental strain of Cdr1p- and Cdr2p-GFP strains (azole resistant)	[22]
DSY3098		DSY3040	0.25	Cdr1p-GFP (azole susceptible)	This study
DSY3108		DSY3041	64	Cdr1p-GFP (azole resistant)	This study
DSY3091		DSY3040	0.25	Cdr2p-GFP (azole susceptible)	This study
DSY3092		DSY3041	64	Cdr2p-GFP (azole resistant)	This study

RNase H^- Reverse Transcriptase (Invitrogen) were added to each sample, followed by incubation at 42°C for 1 h after 5 min at 94°C to inactivate the reverse transcriptase. The cDNA was stored at –20°C until further use.

Gene expression analysis

Gene expression analyses were carried out using the real-time PCR system (LightCycler® 480, Roche) and 96-well microtiter plates. The expressions of the following genes were analyzed: *CDR1*, *CDR2*, *MDR1* and *ERG11*. Primers and probes (TIB Molbiol, Berlin, Germany) are listed in Table 2. Each cDNA sample was analyzed in duplicate and normalized against expression of the *C. albicans* reference gene *ACT1*. Each 25 μl PCR reaction contained: 10 × PCR buffer (20 mM Tris-HCL (pH 8.4), 500 mM KCL), 4.5 mM $MgCl_2$, 200 μM dNTP, 400 nM sense and 400 nM antisense primer, 120 nM probe with a reporter and quencher dye, and 0.75 U of platinum *Taq* DNA polymerase (Invitrogen). Finally, 1 μl of template cDNA was added to each PCR reaction. PCR conditions were: 3 min at 95°C to denature the template and activate the polymerase, followed by 45 cycles of 95°C for 15 s, 60°C for 30 s (primer/probe annealing) and 72°C for 30 s (extension). The crossing point (C_P) values were calculated using the LightCycler® 480 Software (Roche). Relative quantification was performed applying the ΔC_T method [15]. The whole experiment, from culturing the isolates in YPD broth to gene expression analyses, was carried out on two different occasions.

β-galactosidase (lacZ) reporter system

The lacZ reporter system for quantifying *MDR1* and *CDR2* expression has been described elsewhere [20]. To generate *MDR1–lacZ* and *CDR2–lacZ* fusions used in drug induction assays, the 999 bp *MDR1* and 903 bp *CDR2* promoters were first amplified by PCR using the Taq DNA polymerase (Roche) with primers MDR1-5′-999-KPN and MDR1-3′-PST or primers CDR2-KPN and CDR2-PST. The generated fragments were cloned into pAU36 after digestion with *Kpn*I and *Pst*I [21]. The resulting plasmids (pBR1 and pDS295) were digested with either *Nru*I for the *MDR1–lacZ* fusion or *Sna*BI for the *CDR2–lacZ* fusion prior to transformation into *C. albicans*, which enabled their integration at the *MDR1* and *CDR2* loci. Both fusions were integrated by double crossover at the *MDR1* and *CDR2* loci of a *cdr1*Δ/Δ mutant *C. albicans* strain.

Measurement of lacZ reporter activities. Cells were grown overnight in 3 ml of YEPD at 30°C under constant agitation. Cultures were then diluted in 5 ml of fresh medium to obtain a cell density of approximately 5×10^6 cells/ml and were regrown under agitation until the density reached 1.5×10^7 cells/ml. At this point, 1 ml aliquots from drug-unexposed cultures were centrifuged at 4°C for 5 min at 5,500 rpm.

Exposure to cytotoxic drugs. At various time intervals, *C. albicans* strains were exposed to three fixed concentrations (10, 20, 40 μg/ml) of different cytotoxic drugs at 30°C under constant agitation. Benomyl was used as positive control for *MDR1* induction and was measured by *MDR1-lacZ* reporter. Cell density was measured after drug exposure, and cultures

Table 2 Primers and probes used in this study.

Primers/probes	Sequence
CDR1-Hi	5′-GCGCAAGCTTACATCAGAAATTCCTTATCAAGTT
CDR1-SMA1	5′-AACTGTTTACCCGGGTTTCTTATTTTTTTTCTCTCTGTTACC
CDR1-Kp	5′-GCGCGGTACCTACACAATTTTGCAACGGAAGAGTCGG
CDR1-SMA2	5′-AATAAGAAACCCGGGTAAACAGTTTGTTTTTTGACATGGTGG
CDR2-Hi	5′-GCGCAAGCTTATGTACAGATGTAATCCATTCACT
CDR2-SMA1	5′-AGACCCCATCCCGGGTTTTTTCATCTTCTTTTCATTACC
CDR2-Kp	5′-GCGCGGTACCAAGTTGTTTTAACTGGGACCCTGC
CDR2-SMA2	5′-ATGAAAAAACCCGGGATGGGGTCTTATTTTACAATTAGTATT
CDR1 Pr1	5′-AGGAATCGACGGATCAC
CDR1 Pr2	5′-GAAAGAGAACCATTACCAGG
CDR1 probe	5′-TTCTCGCAACACCATACCTCAC
CDR2 Pr1	5′-GAAATATTGTCGCTTTATGAAGCTAGAC
CDR2 Pr2	5′-GTAGCAATAGTGGTTGTAACAGCACC
CDR2 probe	5′-TTACCCGTCAAGTTACTCATGACCATGTCC
MDR1 Pr1	5′-GAATATAAATAAAGGCAGCAATGAC
MDR1 Pr2	5′-GGTAACGACAGAATCACAAGTG
MDR1 probe	5′-CATTAGAAATCACCGTTATGGAACCAGTT
ERG11 Pr1	5′-ATGGCTATTGTTGAAACTGTCATTG
ERG11 Pr2	5′-CCCTTTACCGAAAACTGGAGTAG
ERG11 probe	5′-CATGACCTTTTGGACCTAAATAAACCGTC
ACT1 Pr1	5′-TTCATTGTAAAAAGTGTGATGC
ACT1 Pr2	5′-ATGGACGGTGAAGAAGTT
ACT1 probe	5′-ATCGATAACGGTTCTGGTATGTGTAA

were centrifuged at 4°C and 5,500 rpm for 10 min. The cell pellets were washed with 1 volume Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$) and centrifuged at 4°C for another 10 min. The washed cell pellets were resuspended in 150 µl of Z buffer supplemented with 38.6 mM mercaptoethanol. After adding 50 µl of chloroform and 20 µl 0.1% SDS, cells were disrupted by vortexing. Aliquots preheated at 30°C after adding 700 µl ONPG (1 mg/ml; Sigma) in Z buffer supplemented with mercaptoethanol. The mixtures were incubated at 37°C until they turned yellow (20 min to 3 h). The enzyme was inactivated by adding 500 µl of 1 M Na_2CO_3. The mixtures were centrifuged at 13,000 rpm for 10 min, and the OD_{420} was read. The lacZ activities were calculated using the following equation: lacZ activity (Miller units) = $(1000 \times OD_{420})/(t \times v \times OD_{600})$, where t is the time of reaction expressed in min and v is the culture volume assayed in ml. The experiment was repeated three times.

GFP fusion protein assay

Green fluorescent protein (GFP) was fused to *CDR1* and *CDR2* ORF constructs introduced into azole-sensitive and azole-resistant *C. albicans* isolates. Cdr1p- and Cdr2p-GFP fusions were constructed by insertion of a SmaI site replacing the stop codons of each ORF. For the Cdr1p-GFP fusion, *CDR1* PCR fragments corresponding to the C- terminal end of the protein were generated from a cloned *CDR1* in pDS243 with primer pair CDR1-Hi and CDR1-SMA1 and primer pair CDR1-Kp and CDR1-SMA2. Primer sequences are listed in Table 2. The two PCR fragments were next used in a sewing PCR with the external primers CDR1-Hi and CDR1-Kp and cloned into pDS505 after HindIII and KpnI digestion. pDS505 is based on pMTL21 and contains the *C. albicans URA3* gene at the SacI site. The resulting plasmid (pDS605) was containing a SmaI site into which the GFP ORF was introduced in frame with the C-terminal end of Cdr1p. The resulting plasmid, pDS610, was linearized with NcoI and used to transform *C. albicans* strains DSY3040 and DSY3041, which are Ura$^-$ derivatives of the azole-susceptible and azole-resistant strains DSY294 and DSY296, respectively [22]. The resulting strains were DSY3098 and DSY3108, into which the Cdr1p-GFP fusion is in-frame insertion at the *CDR1* genomic locus.

The same strategy was used for the Cdr2p-GFP fusion. *CDR2* PCR fragments corresponding to the C-terminal end of the protein were generated from a cloned *CDR2* in pDS246 with primer pair CDR2-Hi and CDR2-SMA1 and primer pair CDR2-Kp and CDR2-SMA2. Primer sequences are listed in Table 2. The two PCR fragments were next used in a sewing PCR with the external primers CDR2-Hi and CDR2-Kp and cloned into pDS505 after HindIII and KpnI digestion. The resulting plasmid (pDS685) was containing a SmaI site into which the GFP ORF was introduced in frame with the C-terminal end of Cdr2p. The resulting plasmid, pDS706, was linearized with ClaI and used to transform *C. albicans* strain DSY3040 and DSY3041. The resulting strains were DSY3091 and DSY3092, into which the Cdr2p-GFP fusion is in-frame insertion at the *CDR2* genomic locus [22–24].

Transformants were grown with or without doxorubicin at a fixed concentration of 20 µg/ml in the logarithmic phase, and GFP fluorescence was visualized by microscopy. Fluorescence microscopy was performed with a Zeiss Axioplan microscope equipped for epifluorescence microscopy with a 100-W mercury high-pressure bulb and Zeiss filter sets 9 (for GFP). A DX30 digital camera with high resolution (Kappa Messtechnik GmbH, Gleichen, Germany) was used to record images.

Immunoblot

Overnight cultures of *C. albicans* isolates in YEPD were diluted in 5 ml of fresh medium to an OD_{600} of 1.0 and incubated at 30°C until growth reached an OD_{600} of 2.0.

Cells were then incubated for 90 min with or without doxorubicin (20 μg/ml) or for 30 min with and without fluphenazine (20 μg/ml) as a positive control. Cultures were then pelleted, resuspended in 1 ml H_2O and lysed for 10 min at 4°C by adding 150 μl Yex (Yeast extraction) lysis buffer (1.85 M NaOH and 7.5% β-mercaptoethanol). Lysis products were precipitated for 10 min at 4°C with 50% trichloroacidic acid solution and spun down for 5 min at 4°C. Pellets were resuspended in sample buffer (40 mM TrisHCL pH 6.8, 8 M urea, 5% SDS, 0.1 mM EDTA, 1% β-mercaptoethanol, 0.1 g/l bromophenol blue) and neutralized with 100 mM Tris pH 9.5. Proteins were incubated for 30 min at 37°C and spun down to remove cellular fragments. Proteins were stored at –20°C until further use.

Proteins were denatured for 10 min at 37°C before loading in SDS-PAGE (10%). The prestained SDS-PAGE® (BioRad) molecular weight standard was used to assess protein molecular weight. The migration was performed with running buffer (Tris 1.5 g/l, glycine 7.2 g/l, SDS 0.5 g/l) at 150 V for 2 h.

The migration gels and nitrocellulose transfer membranes (Whatman® Protran®) were soaked in Towbin buffer (Tris 25 mM pH 8.3, 192 mM glycine, 15% methanol, 0.01% SDS). The transfer was performed for 1 h at 75 V. The transferred membranes were blocked for 30 min in a blocking solution (5% powdered milk, PBST 0.1% (137 mM NaCl, 2.7 mM KCl, 21.7 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 0.2% Tween 20). After transfer, remaining proteins in the polyacrylamide gels were visualized by Coomassie Blue staining to control equal loading of proteins of each sample. Blocked membranes were then incubated separately with polyclonal rabbit primary antibodies α-Cdr1p, α-Cdr2p or α-Mdr1p (diluted 1:1000 in the blocking solution). The membranes were washed three times for 5 min in PBST, incubated overnight with goat anti-rabbit polyclonal secondary antibody coupled to horseradish peroxidase (Dako) (diluted 1:3000 in the blocking solution) and then washed 3 times for 5 min in PBST. Finally, the membranes were incubated for 3 min with the detection solution (50% Lumiglo reagent A, 50% Peroxide reagent B (Pierce). The luminescence signals were detected by film autoradiography (Amersham Hyperfilm™ MP).

Determination of minimal inhibition concentrations

MIC values were determined by two methods. One protocol (macrodilution method) followed the recommendations of document M27-A of the Clinical and Laboratory Standards Institute [25]. The other method (microdilution method) differs from recommendations of the macrodilution method and was used to examine MIC values in the presence of doxorubicin [7]. For the microdilution protocol overnight cultures of *C. albicans* strains were diluted 1:100,000 in 200 μl of fresh YEPD with different fluconazole concentrations (0.0625–128 μg/ml) and with or without doxorubicin (20 μg/ml) in 96-well plates. Plates were incubated at 34°C for 24 h, and the OD was measured at 600 nm. The MIC_{50} to fluconazole was defined as the ability of a strain grown with fluconazole to reach 50% of the growth of the same strain grown in YEPD alone. The experiment was performed in triplicate.

Statistical methods

Statistical significance (PCR results) was calculated using the Mann-Whitney test. The results are expressed as means ± standard deviation and were considered significant at $P < 0.05$.

Results

Doxorubicin induces CDR1 and CDR2 expression in C. albicans

CDR1 and *CDR2* expression was tested at the mRNA level after induction by antineoplastic drugs at therapeutic concentrations. Doxorubicin induced *CDR1* and *CDR2* in both sensitive and resistant strains (Fig. 1). The expression of *CDR1* and *CDR2* was enhanced significantly in all doxorubicin-treated isolates after 90 min. The upregulation persisted or increased after 24 h of induction except F1, F5 and Gu1, in which *CDR2* upregulation after 24 h was not detected (Fig. 1). Most of the isolates showed a 2- to 3-fold expression increase at the transcriptional level and in some cases this difference was even higher.

The highest upregulation after 24 h of induction was observed in the isolate pair Gu1/5, in which Gu5 (resistant strain) already exhibited high *CDR1* expression levels without drug exposure. Noteworthy is the fact that, after doxorubicin exposure, the azole-susceptible strain of this pair (Gu1) showed *CDR1* expression levels comparable to those of the resistant isolate. Moreover, the resistant strain (Gu5), which already exhibits intrinsic high *CDR1* expression in the absence of doxorubicin, showed increased expression principally for *CDR2* (>10-fold expression increase after 24 h doxorubicin exposure) (Fig. 1).

Isolates from the other two individuals (F and B) in which the resistant isolates were primarily characterized by overexpression of *MDR1*, showed a significant induction of *CDR* genes by doxorubicin in both the fluconazole resistant and the susceptible strain (>2-fold increase) (Fig. 1).

Since *CDR1* and *CDR2* are regulated by the transcriptional factor *TAC1,* it is likely that *TAC1* is involved in the process of doxorubicin-mediated induction. Consistently,

when a *tac1*Δ/Δ null mutant was used in our experiments, no significant induction by doxorubicin was observed for both *CDR1* and *CDR2*. In contrast, the parental strain was still able to respond to doxorubicin treatment by upregulation of *CDR1* and *CDR2* (Fig. 2).

Doxorubicin had no effect on *MDR1* or *ERG11* expression in the isolates investigated in this study, which indicated a gene-specific effect of doxorubicin in *C. albicans* (Fig. 1, Fig. 3C).

We also examined cyclophosphamide, another frequently used cytostatic drug in cancer therapy which is no inducer of human P-gp. Conversely to doxorubicin, no effect on the expression of any of the investigated resistance genes could be observed with cyclophosphamide at defined and physiologically achievable concentrations (Fig. 1).

Assessing doxorubicin induction by an alternative method

The real-time PCR results were confirmed by a second method. With a *lacZ* reporter system developed for recording of *CDR2* and *MDR1* expression, an elevation of *CDR2* expression was measured after doxorubicin addition at different concentrations (Fig. 3). The effect was dose and time dependent. We observed that increasing doxorubicin concentrations in the assay resulted in a progressive elevation of the *lacZ* reporter activities. The *lacZ* reporter activities at a fixed doxorubicin concentration (20 μg/ml) reached a plateau after 120–180 min exposure. No effect of doxorubicin on *MDR1* expression using the β-galactosidase reporter system could be observed confirming real-time PCR results (Fig. 3C).

Upregulation of CDR1/2 by doxorubicin correlate with increased levels of Cdr1p and Cdr2p in C. albicans

We addressed the localization of Cdr1p- and Cdr2p-GFP after doxorubicin treatment to correlate elevated mRNA levels with enhanced presence of the protein due to doxorubicin treatment. Cdr1p-fusion protein expressed in *C. albicans* was visualized before and after incubation with doxorubicin. Similar to the real-time PCR results, a clear effect of doxorubicin was observed for the GFP fusion proteins, especially in the azole-susceptible strains. As shown in Fig. 4, an increase of fluorescence was observed after incubation with doxorubicin at therapeutic concentrations in the azole-susceptible isolate DSY3098. In the azole-resistant isolate DSY3098, since the GFP signal is already intrinsically high in the absence of doxorubicin, this drug could not further increase this intensity. No fluorescence enhancement was observed after incubation with cyclophosphamide. Experiments using Cdr2p-GFP showed comparable results with the exception that no fluorescence could be detected in the azole-susceptible isolate DSY3092 in the absence of doxorubicin as opposed to drug-exposed conditions (Fig. 4).

When analyzed with anti-Cdr1p and anti-Cdr2p antibodies, the doxorubicin induction of Cdr1p and Cdr2p could be confirmed for azole-susceptible isolates (isolates F1, B1 and Gu1, Fig. 5), thus again confirming RT-PCR results. In most cases the basal Cdr1p levels of fluconazole-susceptible isolates were enhanced when doxorubicin was present. In contrast, Cdr2p showed no basal expression and was detectable only during exposure of isolates to doxorubicin.

In azole-resistant isolates, the effect of doxorubicin on Cdr1p and Cdr2p levels was less clear. Cdr1p levels in isolates F5 and B5, which show as expected high intrinsic Mdr1p levels, were almost not elevated in the presence of doxorubicin and Cdr2p levels almost absent in the same isolates, which contrasts with RT-PCR results (Fig. 1).

Overexpression of Cdr1/2p by doxorubicin leads to increased fluconazole resistance in C. albicans

MIC values of fluconazole-susceptible isolates B1, F1 and Gu1 were additionally determined either with or without the addition of doxorubicin using the microdilution method. For all three isolates a shift of the MIC_{50} values was observed in the presence of doxorubicin (Fig. 6), indicating a possible influence of doxorubicin in the development of the resistance phenotype.

However, in spite of the clear induction of efflux pumps by doxorubicin most of the isolates incubated with doxorubicin increased but did not reach the fluconazole MIC values of the resistant strains (without doxorubicin).

Discussion

This study addressed the question whether cytostatic drugs (doxorubicin and cyclophosphamide) could affect azole resistance in *C. albicans*. We tested major genes responsible for the development of azole resistance in *C. albicans* (*CDR1/2*, *MDR1* or *ERG11*) and used two different cytotoxic drugs, one including a P-gp substrate (doxorubicin) and the other a P-gp non-substrate (cyclophosphamide). A significant effect of doxorubicin at therapeutic concentrations was observed for *CDR1* and *CDR2*. Doxorubicin did not have any effect on investigated genes other than *CDR1/2*. On the other hand cyclophosphamide did not induce any of the tested drug resistance genes.

The induction of *CDR1/2* was detected at the transcriptional and translational level in azole-susceptible isolates, however it was not as prominent in azole-resistant isolates. We observed that doxorubicin enhanced expression of *CDR1/2* in the azole-resistant isolates F5 and B5, in which resistance is known to be acquired by *MDR1* upregulation.

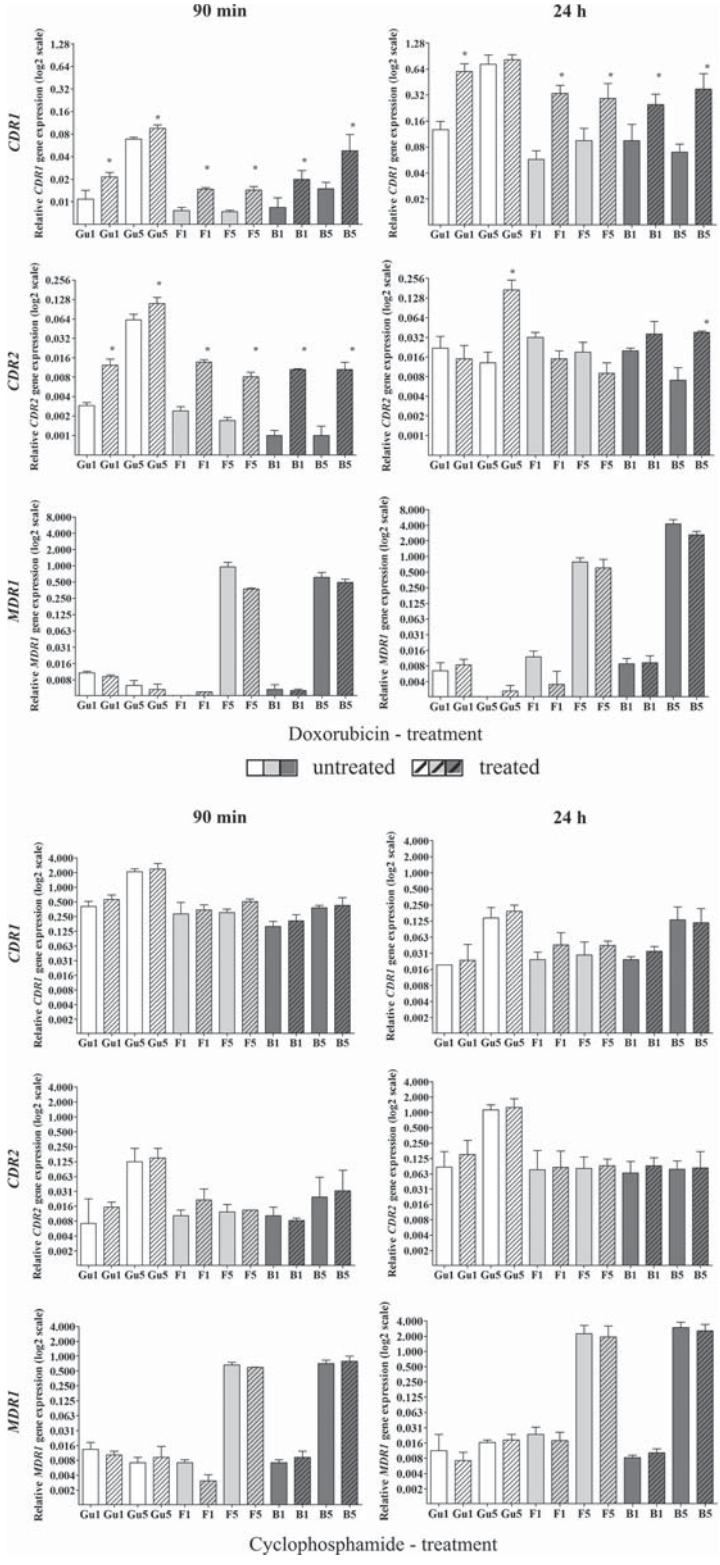

Fig. 1 Results of *CDR1*, *CDR2* and MDR1 gene expression analyses. Clinical isolates were cultivated in the presence or absence of 20 μg/ml doxorubicin or cyclophosphamide, respectively. Gene expressions were normalized against *ACT1*. Left panel: Gene expressions after 90 min of drug exposure. Right panel: Gene expressions after 24 h of drug exposure. The y-axes are presented in log2 scale. Results are means of two biological replicates (four technical replicates in total) ± standard deviation. $*P < 0.05$ compared to untreated control (Mann-Whitney test).

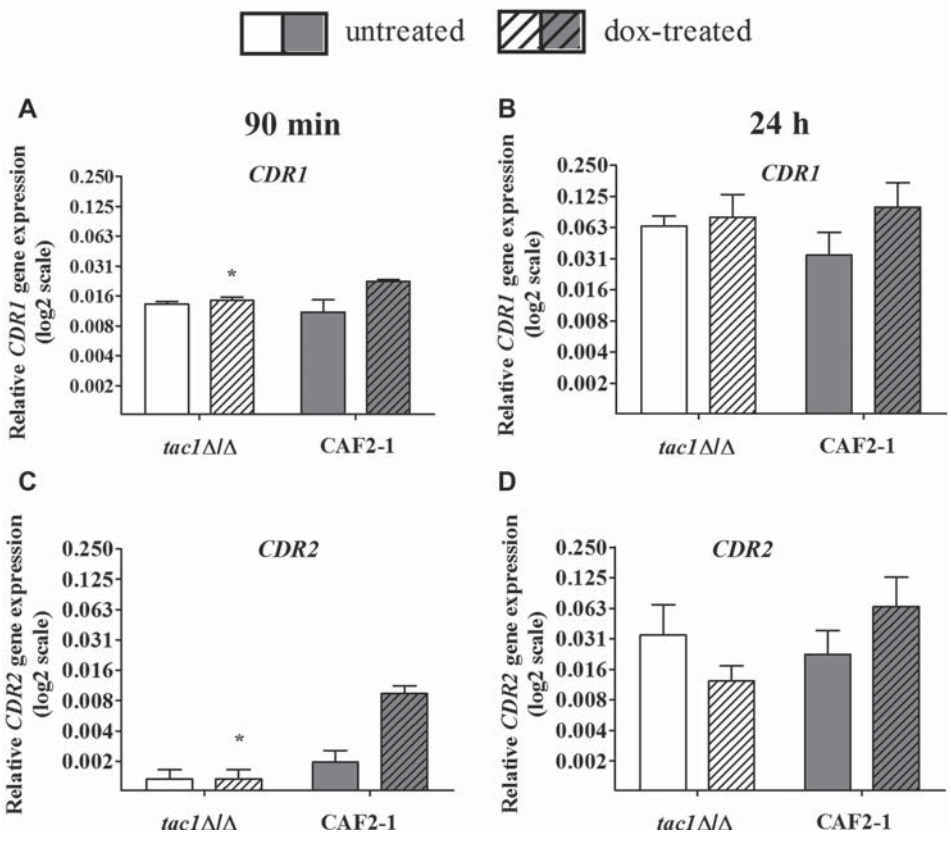

Fig. 2 *CDR1* and *CDR2* gene expression after exposure (20 μg/ml doxorubicin) of the *tac1*Δ/Δ mutant and the parental strain CAF2-1. Gene expressions were normalized against *ACT1*. (A) Relative *CDR1* expression after 90 min and (B) 24 h doxorubicin treatment. (C) Relative *CDR2* expression after 90 min and (D) 24 h doxorubicin treatment. The y-axes are presented in log2 scale. Results are mean of four replicates ± standard deviation. $^*P < 0.05$ compared to doxorubicin-treated CAF2-1.

Curiously, this was not verified at the translational level (Fig. 5). Several possibilities may explain this discrepancy. Besides the different sensitivities between the methods used (RT-PCR vs. immuno-detection), a possible incompatibility between the presence of both elevated Mdr1p and Cdr1p/Cdr2p levels in the plasma membrane of doxorubicin-exposed cells could exist. This hypothesis should be tested by future experiments.

The induction of *CDR1/CDR2* by doxorubicin can therefore be added to the list of other substances mediating

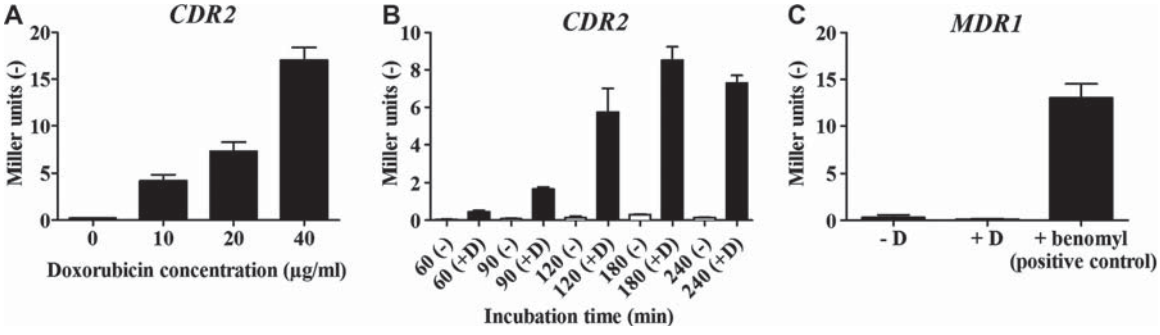

Fig. 3 *MDR1* and *CDR2* expression after doxorubicin treatment by a β-galactosidase reporter system. (A) *CDR2* induction by doxorubicin is concentration dependent. Measurements were performed after 2 h of incubation. (B) Induction of *CDR2* by doxorubicin (20μg/ml) is time-dependent. (-) and (+ D) indicate absence and presence of doxorubicin for the given incubation times. (C) The *MDR1-lacZ* reporter system was incubated with doxorubicin. (- D) and (+ D) indicate absence and presence of doxorubicin. Benomyl was used as positive control. Results of all experiments are means of three replicates ± standard deviation.

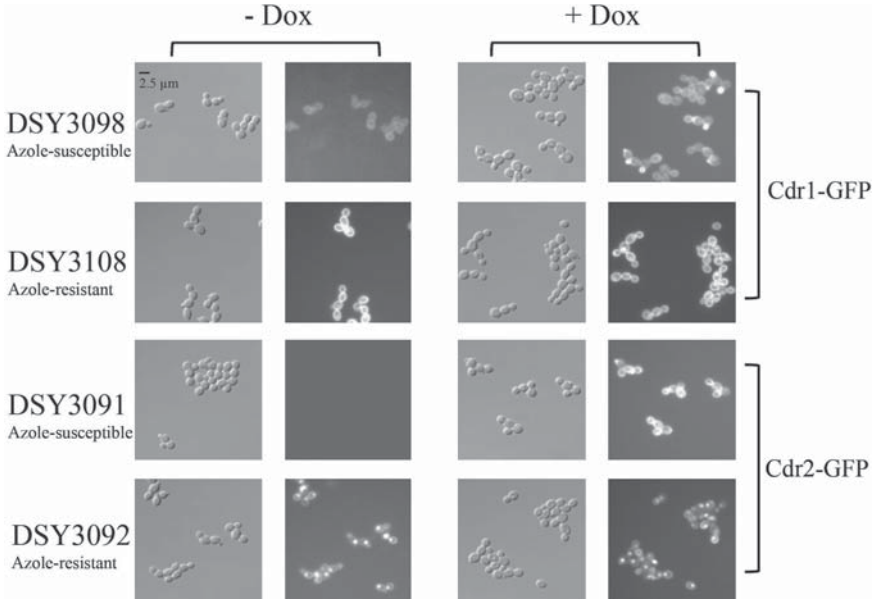

Fig. 4 Localization of Cdr1p- and Cdr2p-GFP fusion proteins in *C. albicans* isolates in absence or presence of 20 µg/ml doxorubicin.

this effect including, for example, bioactive compounds such as steroids, drugs such as fluphenazine or antifungal agents like terbinafine [24,26]. Exposure of *C. albicans* to these substances results in transient upregulation of *CDR1/2*, most probably because these substances are themselves substrates for the transporters. Upon transporter induction, the substances are effluxed by *C. albicans*, thus decreasing their induction potential [26]. It is still unknown whether doxorubicin is a substrate for Cdr1p and Cdr2p. Intriguingly, *CDR1/2* expression is elevated even after 24 h of doxorubicin exposure (Fig. 1) and thus suggests that this substance may be weakly effluxed in *C. albicans*.

Manoharlal and colleagues reported recently that *CDR1* expression in azole-susceptible clinical isolates of *C. albicans* is transient and reversible. It depends mainly on increased transcriptional activation of the gene which is under the control of *TAC1*. Increased stability of *CDR1* mRNA in azole-resistant isolates can also contribute to

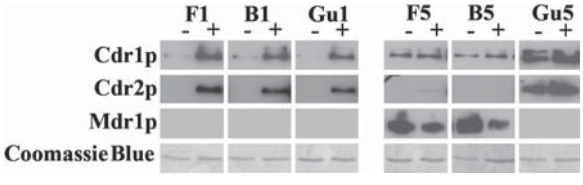

Fig. 5 Cdr1p, Cdr2p and Mdr1p detection after incubation with doxorubicin for 90 min. The left panel shows results of fluconazole-susceptible *C. albicans* isolates. The right panel shows the results of fluconazole-resistant *C. albicans* isolates. (+) doxorubicin induction (20 µg/ml); (-) untreated control. The Coomassie Blue stained signals were revealed after protein transfer on the nitrocellulose membrane and show that equivalent protein amounts were loaded in each sample.

resistance [27]. Here we showed that doxorubicin could increase the expression of *CDR1* and *CDR2* in a *TAC1*-dependent manner (Fig. 2), which is consistent with the critical role of *TAC1* in the regulation of both genes. Transient upregulation of multidrug transporters in *Saccharomyces cerevisiae* by drugs is thought to be the result of activation steps in transcriptional activators via phosphorylation and recruitment of co-activators [28,29]. It is likely that activation of *TAC1* by doxorubicin may follow similar steps. However, other transcriptional elements may participate to *CDR1* and *CDR2* regulation. For example, our study showed here that basal *CDR1* and *CDR2* expression is increased after 24 h incubation in the absence of *TAC1* (Fig. 2), thus highlighting alternative regulatory circuits of these transporters.

Induction of multidrug transporters is also a major clinical problem in treating human cancers with conventional chemotherapeutic drugs. The phenomenon of multidrug resistance (MDR) is mediated in most cases by overexpression of the human efflux pump P-glycoprotein (P-gp). Recently some studies proposed that this protein could also play an important role in delaying apoptosis [26]. MDR-associated cytotoxic drugs possess the ability to modulate transcription levels of the *HsMDR1* gene (Homo sapiens *MDR1* gene coding for human P-gp). Several drug-responsive elements have been identified within the *HsMDR1* gene promoter and the inducibility of the gene via *HsMDR1* promoter sequences by classical MDR-associated cytostatic drugs such as doxorubicin, vincristine or actinomycin D has been be shown. This phenomenon is responsible for MDR during chemotherapy [30].

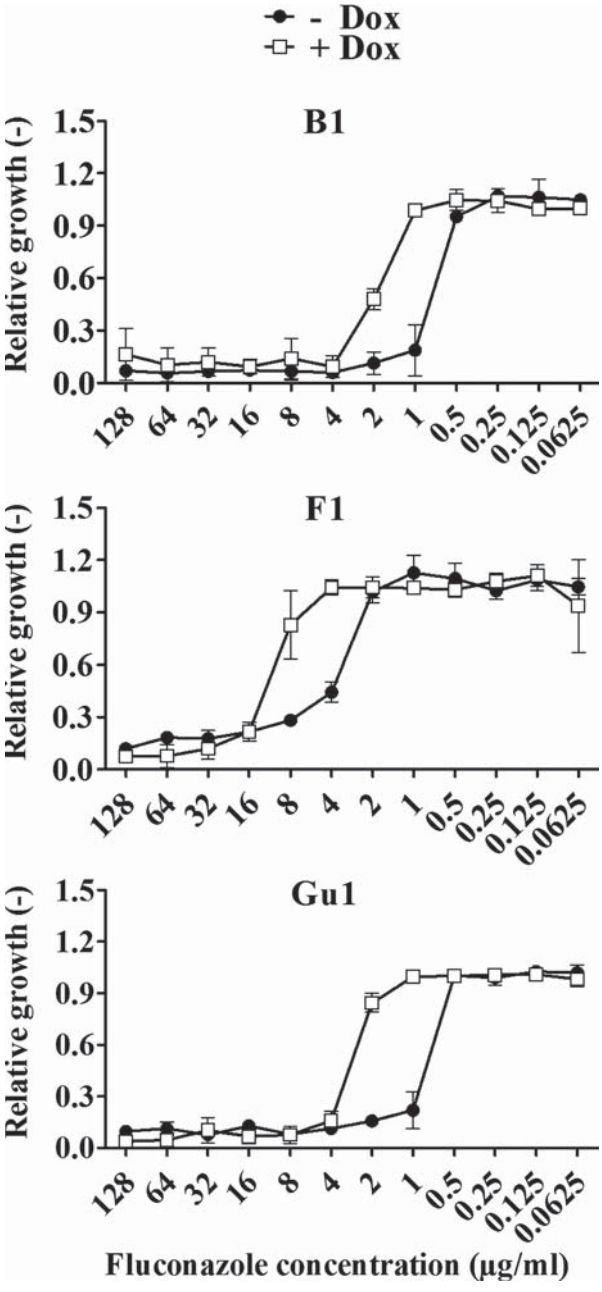

Fig. 6 MIC values of fluconazole-susceptible isolates. The MIC values for fluconazole were determined in the presence and absence of 20 µg/ml doxorubicin. Means of three biological replicates ± standard deviation are shown.

One of the consequences of the induction of multidrug transporters by specific compounds is that they antagonize the activity of antifungal agents [31]. Development of resistance can also be induced *in vitro* by adding fluconazole to a culture suspension containing *C. albicans* strain ATCC36082 after serial passaging in the drug-containing medium [32]. The resistance mechanisms seem to be inducible by a fluconazole therapy and reversible after stopping of this therapy [32,33]. Most of the strains investigated here incubated with doxorubicin increased their fluconazole MICs. Even if this MIC increase did not reach the MIC values of resistant strains, the apparent antagonism between doxorubicin and fluconazole could be of clinical relevance. When antifungal treatment of *Candida* infections occurs simultaneously with anticancer chemotherapy, then it is possible that treatment efficacy could be compromised. Experimental infection models could be used in the future to address this hypothesis.

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