Rifampicin induces MDR1 expression in Candida albicans

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Objectives: Overexpression of efflux pumps such as MDR1 has been identified as an important mechanism contributing to fluconazole resistance in Candida albicans. This phenomenon is frequently observed in fluconazole-resistant strains isolated from AIDS patients treated with various pharmaceuticals. Therefore, we hypothesized that some of these compounds might influence the expression of genes responsible for fluconazole resistance.

Methods: We examined a variety of clinically relevant compounds for their in vitro effects on MDR1 expression with a C. albicans reporter strain containing a transcriptional fusion of the MDR1 promoter (MDR1P) with the gfp gene. Activation of the MDR1 promoter and subsequent green fluorescent protein production was determined by fluorescence microscopy and flow cytometry. Additionally, MDR1 transcription was confirmed and quantified by RT–PCR analysis, followed by Mdr1p detection by western blot. Finally, the effect of a selected agent on resistance to fluconazole was tested by checkerboard titration of both substances.

Results: Of 15 compounds tested, only rifampicin induced a rapid and dose-dependent increase in MDR1 expression (up to 122-fold induction), whereas structurally related molecules such as rifabutin and rifamycin were not active. Induction of MDR1 expression upon rifampicin exposure was also observed in 10 blood culture isolates. In contrast, rifampicin exposure did not markedly affect the expression of the transporters CDR1 and CDR2. Increased MDR1 expression was accompanied by elevated MICs for fluconazole after exposure of C. albicans to rifampicin, whereas Mdr1p expression was only moderately induced.

Conclusions: Out of the compounds examined, only rifampicin specifically induced MDR1 expression in all C. albicans strains tested. Rifampicin may play a general role in signal transduction or another means of modulation of gene expression in C. albicans.

Keywords: MFS transporter, ABC transporter, gene expression

Introduction

During the last decade, invasive infections by Candida albicans have emerged as an increasing problem especially in haematological and oncological patients as well as in patients with AIDS. Despite the urgent need for antifungal agents, only very few substances are established in treatment of systemic fungal infections. Azoles, especially fluconazole, have arisen as first-line antimycotics for prophylaxis and treatment of fungal infections. As the number of patients treated with fluconazole has increased over the last 15 years, fluconazole resistance has emerged as a major problem.

Overexpression of efflux pumps leading to a decreased intracellularazole concentration has been identified as an important mechanism contributing to fluconazole resistance in C. albicans. Efflux pumps are multifunctional membrane proteins involved in diverse physiological functions and export of endogenous and exogenous toxicants. Two families of membrane proteins, the ATP-binding cassette (ABC) and the major facilitator superfamily (MFS) transporters, play a major role in azole drug resistance. ABC transporters as primary active transporter systems utilize the energy of ATP hydrolysis for transport of substrates across cell membranes, whereas transport of substrates by MFS transporter (secondary active) is driven by the proton-motive force. These multidrug transporters are characterized by their ability to bind to a broad range of structurally unrelated drugs and expression of multidrug transporters can also be induced by a variety of different compounds. Overexpression of genes encoding ABC (Cdr1p, Cdr2p) or MFS (Mdr1p) transport
proteins has been well documented in clinical azole-resistant C. albicans isolates. Development of fluconazole resistance in C. albicans is associated with high cumulative doses of fluconazole, but resistant strains have also been isolated in patients without prior azole exposure. Fluconazole-resistant C. albicans are mainly isolated in AIDS patients and other immunocompromised patients that are frequently treated with diverse antimicrobial drugs. Therefore, it could be assumed that some of these drugs may influence the expression of genes responsible for fluconazole resistance.

To investigate whether drugs used in patients may affect the molecular mechanisms that promote resistance of C. albicans to azoles, we examined a variety of different clinically relevant compounds for their in vitro effects on MDRI expression in C. albicans.

Materials and methods

C. albicans strains and growth conditions

In this study, a C. albicans wild-type strain (SC5314) and a C. albicans reporter strain (CAG48A) were used. The reporter strain CAG48A was kindly provided by J. Morschhäuser, Institute for Molecular Infection Biology, Würzburg, Germany. It is a derivative of the C. albicans strain CAI4, which expresses a single copy of the green fluorescent protein (GFP) reporter gene from the endogenous MDRI promoter (MDRI/mdr1::PMDRI-GFP::URA3, J. Morschhäuser, personal communication). Additionally, 10 C. albicans strains, isolated from blood cultures, were analysed. The strains were kept as frozen stocks in glycerol at 80 °C. Aliquots were streaked on yeast-dextrose agar plates (5 g of yeast extract, 10 g of peptone, 20 g of dextrose, 15 g of agar and 40 mg gentamicin per litre), incubated at 30 °C overnight and subcultured in yeast nitrogen base (YNB) liquid medium (0.67% YNB and 0.5% dextrose) at 30 °C. Cultures were diluted in 25 mL YNB medium to an optical density at 600 nm (OD600) of 1 and were incubated at 30 °C for 4 h with gentle shaking.

Reagents

All reagents were obtained from Sigma. For broth microdilution, fluconazole was obtained from Phast-GmbH (1271700, Homburg, Germany). Compounds were dissolved in ethanol (metronidazole and spiramycin), DMSO (rifampicin, albendazole and praziquantel) or water (all others).

Induction assays

Test compounds (Table 1) were added to the cultures and were incubated as described earlier. Aliquots of the cultures were taken after 4 h and spotted on microscope slides. Fluorescence was detected with a Leica DM RE HC microscope equipped for epifluorescence microscopy and with a fluorescein-specific filter set (I3). The degree of fluorescence was compared with the fluorescence induced by benomyl (50 mg/L). Candida cells treated for 4 h with rifampicin (5, 10, 20 and 40 mg/L) were washed twice and 1 × 10^6 cells/mL per sample were analysed by flow cytometry (fluorescence activated cell sorting, FACS) on a FACSCalibur flow cytometer (BD Bioscience, Heidelberg, Germany). Controls were performed with untreated, benomyl- (50 mg/L) or amphotericin B- (1 mg/L) treated cells. Data were analysed with CELLQuest Pro Software (BD Bioscience). Cells were gated on an appropriate size to exclude cell debris.

RNA isolation

Total RNAs were extracted from C. albicans cells by use of the MasterPure™ Yeast RNA Purification Kit (EPICENTRE, Madison, WI, USA). Briefly, treated cultures (1–5 mL) were centrifuged and pellets were incubated at 70 °C for 15 min with 300 µL of extraction reagent containing proteinase K. After cooling on ice, 175 µL of RNA was isolated. For testing mRNA stability, Candida cells were treated for 4 h with benomyl (50 mg/L) or rifampicin (40 mg/L) then washed and incubated in fresh medium (YNB) up to 8 h. Aliquots were taken prior to induction and after 4 h rifampicin exposure. After removal of the inducing agent, additional samples were taken at 0, 0.5, 2, 4 and 8 h incubation in YNB followed by RNA isolation and RT–PCR.

Table 1. Effect of various compounds on MDRI induction

<table>
<thead>
<tr>
<th>Group</th>
<th>Compound</th>
<th>MIC (mg/L)</th>
<th>Concentrations tested (mg/L)</th>
<th>Relative MDRI induction</th>
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</thead>
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<tr>
<td>MDRI-inducer</td>
<td>benomyl</td>
<td>&gt;250</td>
<td>10, 15</td>
<td>+</td>
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<td>Antibiotics</td>
<td>gentamicin</td>
<td>&gt;250</td>
<td>10, 15</td>
<td>0, 0</td>
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<td></td>
<td>penicillin</td>
<td>&gt;250</td>
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<td></td>
<td>erythromycin</td>
<td>12.5</td>
<td>4, 8</td>
<td>0, 0</td>
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<tr>
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<td>10, 20, 50</td>
<td>0, 0, 0</td>
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<td>250</td>
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<td>10, 50</td>
<td>0, 0</td>
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<td></td>
<td>rifamycin B</td>
<td>&gt;250</td>
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<tr>
<td></td>
<td>rifampicin</td>
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<td>5, 10, 20, 40</td>
<td>0, (+), (+), (+)</td>
</tr>
<tr>
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<td>rifabutin</td>
<td>125</td>
<td>10, 40</td>
<td>0, (+)</td>
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<tr>
<td></td>
<td>fusidic acid</td>
<td>&gt;250</td>
<td>15, 30</td>
<td>0, 0</td>
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<tr>
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<td>12.5</td>
<td>2, 4</td>
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<td>0, 0</td>
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<td>thiabendazole</td>
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<td></td>
<td>DMSO</td>
<td>250</td>
<td>50, 100</td>
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*CAG48A, a derivative of C. albicans strain CAI4, harbouring a single copy of the gfp reporter gene under control of the native MDRI promoter was used as a reporter strain and incubated with the various compounds as described in the Materials and methods section. The degree of fluorescence was determined by microscopy and compared with the fluorescence induced by benomyl (50 mg/L) and categorized as 0 = no fluorescence, (+) = lower numbers of fluorescent cells, + = fluorescence as benomyl control or ++ = higher numbers of fluorescent cells.

For RT–PCR analysis, strains were incubated in YNB medium with rifampicin (40 or 320 mg/L) or benomyl (50 mg/L). Cells were harvested at indicated time points of drug exposure up to 4 h before RNA was isolated. For testing mRNA stability, Candida cells were treated for 4 h with benomyl (50 mg/L) or rifampicin (40 mg/L) then washed and incubated in fresh medium (YNB) up to 8 h. Aliquots were taken prior to induction and after 4 h rifampicin exposure. After removal of the inducing agent, additional samples were taken at 0, 0.5, 2, 4 and 8 h incubation in YNB followed by RNA isolation and RT–PCR.
quantified spectrophotometrically. RNA samples were stored at
−80°C or were used immediately.

cDNA synthesis and RT–PCR
cDNA was synthesized with oligo(dT)-primers (New England Biolabs, Frankfurt, Germany) and SuperscriptIII reverse transcriptase (Invitrogen, Karlsruhe, Germany), using 1 μg of total RNA. Controls examined *C. albicans* elongation factor 1 (EF1β) as a housekeeping gene, which contains an intron of 365 bp. Absence of genomic DNA was verified by a single intronless PCR product of EF1β. PCRs were performed according to standard protocols with Taq DNA polymerase (Roche, Mannheim, Germany) and MDR1-specific primers complementary to nucleotides +827 to +851 and +1201 to +1225 of MDR1 (MDR1-fd: TTT GGT TCA TGT GTA TCA TTT CTG G; MDR1-rv: GAA TAT AAA TAA AGG CAG CAA TGA C), CDR1-specific primers complementary to nucleotides +3506 to +3527 and +3985 to +4005 (CDR1-fd: GGT ATT ACC ATT GGA TTT GCC G; CDR1-rv: GAA GAA TCT AAG GCA TGG CC) and CDR2-specific primers complementary to nucleotides +4570 to +4589 and +4979 to +4998 (CDR2-fd: ACA AAG TCA AAT GTT GGC GG; CDR2-rv: TTA GGA CCA GCC AAT ACC CC). Cycling conditions were 94°C for 30 s, 52°C for 30 s and 72°C for 40 s (28 cycles) for MDR1, 94°C for 30 s, 52°C for 30 s and 72°C for 60 s (21 cycles) for EF1β, 94°C for 30 s, 63°C for 30 s and 72°C for 60 s (27 cycles) for CDR1 and 94°C for 30 s, 63°C for 30 s and 72°C for 60 s (30 cycles) for CDR2. Only samples from the exponential phase of PCR amplification were examined. Equivalent volumes of PCR product were analysed on 1.8% agarose gels stained with ethidium bromide.

For quantitative analysis of gene expression of MDR1 and CDR1, a LightCycler real-time RT–PCR was performed as described by Frade et al. with the following modifications: MDR1-specific primers (MDR1-fd and MDR1-rv) were used as described above employing the manufacturer’s protocols for SYBER green quantification. Cycling conditions for MDR1 were 95°C for 10 s, 54°C for 10 s and 72°C for 16 s (40 cycles). Fluorescence intensities were quantified with the LightCycler Software, using the ‘fit points’ method. Relative quantities of the target genes were standardized on ACT1 expression and normalized on the untreated sample. Data shown are means of two independent experiments performed in duplicate. Error bars indicate standard deviations between these experiments.

Susceptibility testing
MICs were determined by broth microdilution testing according to the standard CLSI broth microdilution protocol (M27-A2). Briefly, cultures were adjusted to an optical density equivalent to that of a McFarland 0.5 standard, then 1:1000 diluted in RPMI 1640 medium buffered with MOPS and 0.2% dextrose. One hundred microlitres of cell suspension was mixed with 100 μL of rifampicin/fluconazole solution in checkerboard titration (fluconazole concentration 0.06–128 mg/L and rifampicin concentration 5–320 mg/L). Microtitre plates were incubated for 48 h at 35°C. MICs were determined spectrophotometrically by 50% decrease in optical density relative to the optical density of the fluconazole-free control well.

Western blot
Proteins were isolated as described by Hiller et al. separated on a 10% SDS gel and transferred to nitrocellulose membranes. Blots were incubated with rabbit-anti-Mdr1p- or anti-Cdr1p-antibodies, respectively (courtesy of D. Sanglard, Lausanne, Switzerland), for 2.5 h. Detection was performed with horseradish peroxidase-conjugated anti-rabbit-antibodies (DakoCytomation, Hamburg, Germany) using ECL chemiluminiscent reagent (Amersham, Uppsala, Sweden).

Statistical analysis
Flow cytometry analysis of the rifampicin effect on MDR1 expression was performed in three independent experiments. Differences between mean values of percentage of GFP-positive cells after rifampicin exposure and in medium controls were compared by Student’s t-test, and significance was defined as P < 0.05.

Results and discussion
The MDR1 gene of *C. albicans* encodes a membrane transport protein of the major facilitator superfamily and mediates resistance to fluconazole and several other, structurally unrelated drugs. Microbial strains do not express large amounts of MDR1 during culture in standard media *in vitro*, but MDR1 is frequently and constitutively expressed in fluconazole-resistant isolates. Compounds such as the benzimidazole fungicide benomyl induce MDR1 transcription in *C. albicans*. Flucanazole-resistant *C. albicans* are mainly isolated in patients who have been frequently treated with diverse antimicrobial and cytostatic drugs. Some of these drugs have been shown to influence expression of CDR1 leading to antagonistic activity against fluconazole. Therefore, we examined a variety of compounds used as antibacterial and antiparasitic drugs for their *in vitro* effects on MDR1 expression. For this purpose, we used a reporter strain containing a transcriptional fusion of the MDR1 promoter (MDR1P) with the gfp gene. The reporter strain was exposed to various drugs and subjected to analysis by fluorescence microscopy and flow cytometry.

Although MDR1 transcription upon benomyl stimulation was detectable within 5 min, production of GFP could be observed microscopically after 1 h, reached a maximum between 2 and 4 h and was stable up to 24 h (data not shown). Therefore, the fluorescence microscopy assay was performed at 4 h after drug exposure for the screening of 15 compounds in order to assess their potential to induce MDR1 expression. For most compounds, at least two concentrations below the MIC representing clinically relevant drug levels have been tested. The results show that only rifampicin, a drug widely used for treatment of bacterial and especially mycobacterial infections, induced a significant activation of the MDR1 promoter of the reporter strain CAG48A (Table 1).

Activation of the MDR1 promoter was further determined by flow cytometry. Rifampicin-treated CAG48A cultures showed significantly higher numbers of fluorescent cells than untreated cultures (P < 0.05). This increase was dose-dependent. At concentrations of 10 mg/L rifampicin, a significantly higher number of fluorescent cells compared with the medium control could be observed. At 20 mg/L, the number of fluorescent cells corresponded to the number of cells stimulated by benomyl (Figure 1). However, while the mean fluorescence intensity was lower upon exposure to rifampicin compared with benomyl, the total number of fluorescent cells was higher in rifampicin (40 mg/L) exposed cells compared with those exposed to...
benomyl, in keeping with the fluorescence microscopy data (Figure 1b).

In order to investigate whether fluorescence signals of the reporter strain upon rifampicin exposure were actually accompanied by *MDR1* mRNA expression, transcription of *MDR1* was confirmed by RT–PCR analysis. The data in Figure 2 show that upon rifampicin exposure, *MDR1* mRNA expression could be observed for the reporter strain CAG48A as well as for the reference strain SC5314. However, compared with the *MDR1* expression upon benomyl exposure,
rifampicin-induced signals appeared weaker in both strains. These results were in keeping with the results obtained by fluorescence microscopy and flow cytometry for the reporter strain.

Rifampicin exposure also resulted in an enhanced transcription of \textit{MDR1}\textsuperscript{5} in blood culture isolates (Figure 3). All of 10 strains tested showed increased \textit{MDR1} signals upon rifampicin (40 mg/L) exposure. Therefore, it can be assumed that \textit{MDR1} expression due to rifampicin treatment is common in \textit{C. albicans} rather than a strain-specific phenomenon.

The activation of \textit{MDR1} expression was clearly evident after a 1 h rifampicin exposure (Figure 4a). Quantification of the \textit{MDR1} gene expression by real-time RT–PCR analysis showed a rise from 10-fold induction at 1 h to 122-fold induction after 2 h (Figure 4b). Previous studies demonstrated that members of the ABC transporter family (\textit{CDR1} and \textit{CDR2}) could be up-regulated in parallel to \textit{MDR1}. In particular, Henry \textit{et al.}\textsuperscript{13} showed that \textit{CDR1} was up-regulated upon exposure to albendazole and sulfadiazine. However, in our study, in contrast to the induction of \textit{MDR1} expression, rifampicin exposure did not affect the expression of \textit{CDR1} and \textit{CDR2} to the same extent. 

\textit{CDR1} transcription was 3–3.5-fold increased 15 min to 1 h after rifampicin exposure and reached a maximum of 5.8-fold induction at 2 h (Figure 4b). \textit{CDR2} mRNA was not detectable.

In order to investigate whether \textit{MDR1} and \textit{CDR1} transcription upon rifampicin exposure resulted in Mdr1p and Cdr1p production, protein preparations of the reference strain (SC5314) were analysed by western blotting with anti-Mdr1p- and anti-Cdr1p-antibodies. While cells exposed to 40 mg/L rifampicin expressed only small amounts of Mdr1p, a significant signal could be observed in cells treated with 320 mg/L rifampicin (Figure 5a). In contrast, expression of Cdr1p, which was also present in the absence of the drug, was only slightly increased in cells exposed to 40 mg/L rifampicin (Figure 5b).

Although an increased \textit{MDR1} expression could clearly be demonstrated, fluconazole MICs of the wild-type strain (SC5314) were not affected by rifampicin concentrations up to 40 mg/L.
80 mg/L. Fluconazole MICs did not exceed 0.5 mg/L. However, rifampicin concentrations of 160 and 320 mg/L caused slightly elevated MICs for fluconazole up to 2 mg/L. In clinical isolates, a 25-fold increase of MDR1 gene expression only moderately elevated the fluconazole MIC from 1 mg/L up to 8 mg/L. In transformed C. albicans strains expressing MDR1 under the control of the ADH1 promoter, MDR1 overexpression resulted in only minor changes of fluconazole MICs. In strain CAG48A, fluorescence intensities of rifampicin-induced cells were much lower compared with benomyl-treated cells, suggesting that MDR1 gene expression induced by rifampicin is not high enough to significantly increase fluconazole MICs. This is in keeping with the results shown in Figure 5 demonstrating a significant production of Mdr1p only at very high rifampicin concentrations.

Rifampicin is a semi-synthetic derivative of rifamycin B, a macrocyclic antibiotic produced by Amycolatopsis mediterranei. Rifampicin, like other rifamycin derivatives, preferentially inhibits the DNA-dependent RNA polymerase of prokaryotes; eukaryotic enzymes are far less sensitive to rifampicin. Rifampicin alone does not show an inhibitory effect on Candida spp. or other fungal species in concentrations up to 200 mg/L. In human cells, rifampicin functions as a potent inducer of the expression of transport proteins, especially the P-glycoprotein belonging to the ABC transporter family and also induces a variety of drug-metabolizing enzymes. It was recently postulated that rifamycins could represent a conserved signalling molecule involved in the regulation of various behaviours in different genera of eukaryotic and prokaryotic cells and viruses. Rifampicin concentrations effective for MDR1 expression were 10 mg/L and higher, and MDR1 expression was dependent on a continued exposure to rifampicin. Within 30 min after withdrawal of rifampicin, MDR1 mRNA was no longer detectable, whereas benomyl exposure resulted in a more prolonged mRNA expression up to 8 h indicating an increased mRNA stability in response to benomyl but not to rifampicin (Figure 6). Up-regulation of the MDR1 gene is influenced by two regulatory elements, H$_2$O$_2$ responsive element (HRE) and benomyl responsive element (BRE). Constitutive high expression of MDR1 is linked to the BRE but not to HRE. It can be hypothesized that the different expression kinetics of MDR1 transcripts in response to benomyl and rifampicin are related to different transactivating factors and that rifampicin acts in a fashion more similar to H$_2$O$_2$ than to benomyl.

In patients, serum levels of rifampicin usually do not exceed 10 mg/L after oral administration of 450 mg; however, tissue levels, especially in lung (4.08 μg/g) and liver (36 μg/g), usually exceed those in serum. Very high drug concentrations between 200 and 350 mg/L accumulate in urine and bile. Moreover, rifampicin-impregnated central venous catheters and other implants have been increasingly used in clinical trials. Such catheters were impregnated with rifampicin solutions up to 30 mg/mL and showed locally released rifampicin amounts up to 35 μg/cm catheter length. It cannot be excluded that in C. albicans biofilms, which have been observed in catheters, rifampicin concentrations exceeding 40 mg/L may occur. Interestingly, during a prospective multicentre trial comparing rifampicin-impregnated central venous catheters with non-impregnated catheters, a significant increase in colonization by Candida spp. was observed. Unfortunately, no MIC studies have been performed on these colonizing Candida strains.

Taken together, the data reported in this study demonstrate that compounds used as antibacterial agents may affect MDR1 expression of C. albicans. In addition, although it is still speculative, it may well be that drugs such as rifampicin might also affect the expression of genes other than MDR1 in C. albicans. As to whether such events might affect virulence of C. albicans needs to be investigated in future studies.

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Transparency declarations

None to declare.

Supplementary data

A colour version of Figure 1 is available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).
Rifampicin induces CaMDR1

References