Transcription factor Efg1 contributes to the tolerance of Candida albicans biofilms against antifungal agents in vitro and in vivo

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We investigated the molecular basis of the tolerance of Candida albicans biofilms to antifungals using the miconazole as a model compound, and translated the resulting data to other antifungals. Sessile cells of C. albicans Δefg1, lacking the transcription factor Efg1, showed increased susceptibility to miconazole, amphotericin B and caspofungin, whereas these sessile cells were equally resistant to fluconazole. The increased sensitivity to miconazole was, at least, partly due to an increased accumulation of miconazole in the cells as compared to wild-type or reintegrant Δefg1(EFG1) sessile cells. By using a rat biofilm model, we further confirmed the role of Efg1 in the tolerance of C. albicans biofilms to miconazole when grown in vivo.

INTRODUCTION

Due to the increasing number of immunocompromised patients, combined with the advances in medical technology, yeast infections have emerged as a major cause of infectious diseases, with Candida albicans being the major pathogen. Upon contact with various surfaces, C. albicans is known to form biofilms, which are a cause of infections associated with medical devices such as indwelling intravascular catheters. Those infections are particularly serious because biofilm-associated C. albicans cells display increased tolerance to a wide spectrum of antifungal drugs, including various azoles, such as fluconazole (MIC50 ≥ 256 mg l−1) and miconazole (MIC50 ≥ 256 mg l−1), and the newer generation of azoles, such as voriconazole (MIC50 ≥ 256 mg l−1) and posaconazole (MIC50 ≥ 64 mg l−1) (Lamfon et al., 2004; Ramage et al., 2009; Katragkou et al., 2008). Miconazole has moderate fungicidal activity against C. albicans biofilms, as 1–10 % sessile C. albicans cells can survive exposure to high doses (2080 mg l−1) of miconazole (Vandenbosch et al., 2010). The basis of this drug resistance of biofilms is not clear. The expression of drug efflux pumps during the early phase of biofilm formation, alterations in membrane sterol composition and the presence of persisters have all been linked to azole resistance of C. albicans biofilms (Mateus et al., 2004; Mukherjee & Chandra, 2004; Bink et al., 2011). Other factors potentially responsible for increased biofilm resistance include (i) expression of resistance genes (e.g. drug efflux pumps), (ii) altered growth rate or metabolic rate of biofilm cells, (iii) drug binding to the extracellular matrix, (iv) changes in membrane composition or (v) higher antioxidative capacities (Seneviratne et al., 2008). Azoles inhibit the 14α-demethylation of lanosterol, resulting in decreased ergosterol levels and the accumulation of toxic sterol intermediates (Kelly et al., 1997; Vanden Bossche et al., 1990). In contrast to fluconazole, miconazole is a fungicidal azole with fungicidal activity against Candida biofilms (Lamfon et al., 2004; Vandenbosch et al., 2010). Besides inhibiting ergosterol biosynthesis, miconazole also induces reactive oxygen species (ROS) in planktonic and biofilm C. albicans cultures (François et al., 2006; Vandenbosch et al., 2010). It is likely that besides ROS accumulation, other mechanisms may be involved in
the fungicidal activity of miconazole against C. albicans biofilms (Vandenbosch et al., 2010). In the present study, we aimed to further unravel the molecular basis of reduced susceptibility or increased tolerance of C. albicans biofilm cells to miconazole. To this end, we determined the miconazole sensitivity of biofilms of C. albicans mutants affected in cyclic AMP signalling, as it was previously reported that the cAMP signalling pathway is involved in the tolerance of planktonic C. albicans cells to miconazole (Jain et al., 2003). In addition, we validated the obtained in vitro data in an in vivo biofilm model, in which we administered miconazole intraperitoneally to rats (Ricicová et al., 2010). We finally demonstrated that by using miconazole as a model antymycotic compound, which is mainly used to treat topical fungal infections (Kyle & Dahl, 2004), these findings can be translated to other antimycotics such as amphoterin B (AmB) and caspofungin (CAS), which are used specifically in combatting systemic fungal infections (Kuhn et al., 2002a).

METHODS

Materials, yeast strains, plasmids and growth media. The C. albicans homozymous deletion mutants used in this study were affected in genes encoding the class III adenyl cyclase (Δcyc35), RAS signal transduction (Δras1), cyclic nucleotide phosphodiesterase 1 (Δpde1) and 2 (Δpde2), and enhanced filamentous growth transcriptional factor (Δefg1) (Davis-Hanna et al., 2008). A second set of C. albicans mutants consisted of Δefg1 (ΔHLC52), the reinteggrant Δefg1ΔΔefg1 (ΔHLC74), and the corresponding wild-type (WT) CAF2 (Lo et al., 1997). Strain DSY1050, in which all major efflux pumps encoding CDR1, CDR2 and MDR1 are inactivated (Perumal et al., 2007), and corresponding WT strain SC5314 were from Gillum et al. (1984). The growth medium used was yeast extract–peptone–glucose (dextrose) (YPD; 1% yeast extract, 2% peptone, 2% glucose).

Planktonic activity assay. Susceptibility testing and determination of MIC of miconazole that inhibits fungal growth by 50% (MIC50) were performed according to the NCCLS M27-A3 protocol (CLSI, 2008) for planktonic cultures.

Biofilm activity assay. The activity of miconazole, AmB, CAS and fluconazole in PBS (2% final DMSO concentration) against 16-h-old C. albicans biofilms (10^7 cells per well) was assessed using the crystal violet (CV) quantification method and by plate counts as described previously (Peeters et al., 2008; Bink et al., 2011; Thevissen et al., 2009). DMSO (2%) in PBS was used as control treatment. Briefly, after incubation for 24 h, biofilms were washed and the percentage of remaining biofilm was calculated as the minimal concentration resulting in 50% eradication of the biofilm (BEC50) or was determined by resuspending biofilms in PBS by vigorous vortexing and determining the fraction of viable biofilm cells by counting the colonies and calculating the number of c.f.u. The percentage of surviving biofilm cells after miconazole treatment was calculated relative to the control treatment (2% DMSO).

Quantitative analysis of the intracellular accumulation of miconazole in C. albicans biofilms. Sixteen-hour-old C. albicans biofilms (10^7 cells per well) were washed with PBS (pH 7.4) and incubated with either 0.6 or 1.2 mM miconazole in PBS for 4 h at 37 °C. After incubation, treated biofilms were resuspended after vigorous vortexing and pooled per five wells. After centrifugation, the cell pellet was washed twice with 500 μl PBS, samples were taken for cell counting in a Thoma counting chamber (Paul Marienfeld) and the pellet was resuspended in 300 μl acetonitrile/PBS (70:30, v/v). Miconazole concentration in the cell lysates was determined using HPLC as described previously (Bink et al., 2010; François et al., 2009) and was normalized to the number of cells in the pellet.

Expression analysis of EFG1 in biofilm cells. Cells of 16-h-old biofilms were collected and washed with physiological saline. Cell disruption, RNA purification, DNase treatment and RT-PCR were performed as described previously (Nailis et al., 2006). After development of forward (5'-CTGCTGGCTCCTCCACT-3') and reverse (5'-CTCGACCGGAAAGCCACGAGC-3') primers for EFG1 and testing for specificity, real-time PCR (CFX96 Real-time System, Bio-Rad) was performed using a Mesa Green qPCR kit (Eurogentec). The expression level of EFG1 in both conditions was normalized using two reference genes (ACT and RPP2B).

In vivo rat biofilm model. A miconazole suspension (20 mg ml⁻¹) for intraperitoneal administration was prepared in MilliQ water containing 11.5% Cremophor EL (Fagron). Methylparaben and propylparaben (Sigma) were added as preservatives (final concentrations 0.5 mg ml⁻¹ and 0.05 mg ml⁻¹, respectively). Control solution without miconazole was also prepared. In vivo biofilm drug susceptibility assays were determined using 1 cm pieces of serum-coated triple-lumen polyurethane catheters (Arrow International) as described previously (Ricicová et al., 2010). Briefly, animals were immunosuppressed 24 h prior to catheter implantation and throughout the whole experimental procedure (up to 9 days). This immunosuppression was accomplished by using dexamethasone (1 mg l⁻¹) supplemented with ampicillin (0.5 g l⁻¹) in the drinking water. Polyurethane catheters were incubated overnight in fetal bovine serum (Sigma) with C. albicans cells (5 × 10^6 cells ml⁻¹) prepared in RPMI1640-MOPS (Sigma). After 90 min of adhesion at 37 °C, catheters were washed twice with PBS to remove unattached cells. The catheter fragments were implanted subcutaneously to the lower back of each rat (nine catheters per rat). Biofilms were allowed to develop for 48 h before miconazole treatment was started. Miconazole or control solution was administered intraperitoneally daily for 7 days to 12 rats (two rats per strain and per treatment), at a dosage of 200 mg kg⁻¹ of body weight. Analysis and quantification of the number of cells per individual biofilm was performed as previously described (Kuchariková et al., 2010).

Statistical analysis. Statistical analysis was performed using an unpaired t-test. Statistical analysis (ANOVA and least significant difference post hoc test) on the log-transformed colony counts of the in vivo experiments was carried out using spss software version 16.0. Differences were considered to be significant if P<0.05. Data of all experiments are represented by the mean ± SD.

Planktonic activity assays were performed as two independent biological repeats, each consisting of four technical repeats. Biofilm activity assays using the CV quantification method were performed as three independent biological repeats, each consisting of two technical repeats. Biofilm activity assays by plate counts were performed as four independent biological repeats, each consisting of two technical repeats. Experiments for quantitative analysis of intracellular accumulation of miconazole in C. albicans biofilms were performed as four biological repeats, each consisting of at least four technical repeats. Experiments regarding expression analysis of EFG1 in C. albicans biofilm cells were performed as three independent biological repeats, each consisting of six technical repeats. In vivo rat biofilm experiments were performed on 18 individual biofilms for each treatment group.
**RESULTS**

**Δefg1 sessile cells are hypersensitive to miconazole**

Note that *C. albicans* strains affected in Efg1 are unable to form real biofilms with a three-dimensional architecture (Ramage et al., 2002). Hence, throughout this study, we have used the term ‘sessile’ cells in case of Δefg1.

We first analysed the miconazole sensitivity of planktonic and biofilm cells of *C. albicans* homozygous null mutants affected in genes involved in the cAMP signalling pathway, namely Δalc35, Δras1, Δpde1, Δpde2, Δefg1, and the corresponding isogenic WT strain CAF2 (Davis-Hanna et al., 2008). Susceptibility testing and determination of MIC50 were performed according the NCCLS M27-A3 protocol (CLSI, 2008) for planktonic *C. albicans* cultures. Biofilm activity assays were performed on 16-h-old *C. albicans* biofilms incubated with various concentrations of miconazole for 24 h following determination of the remaining biomass using the CV quantification method. Table 1 compares the miconazole MIC50 and BEC50 values for planktonic and biofilm *C. albicans* cells, respectively. As shown in Table 1, Δefg1 sessile cells showed increased sensitivity to miconazole (BEC50 = 0.1 mM) compared to sessile cells of the corresponding isogenic WT strain CAF2 (BEC50 > 4.8 mM) and the other tested *C. albicans* strains (BEC50 ranging from 0.8 to > 4.8 mM) (Table 1). Miconazole sensitivity of planktonic Δefg1 cells was not increased compared to the miconazole sensitivity of the other mutants and the WT strain, as represented by their respective MIC50 values (Table 1). These data point to Efg1 as a biofilm-specific factor that determines miconazole sensitivity. To further confirm this observation, we assessed the miconazole sensitivity of a second set of homozygous *C. albicans* mutants, including Δefg1, the reintegrant Δefg1(EFG1) and the corresponding isogenic WT strain CAF2 (Lo et al., 1997) (Fig. 1a). The data presented in Fig. 1(a) demonstrate that in contrast to the WT strain or Δefg1(EFG1) sessile cells, the sessile cells of Δefg1 were sensitive to low doses of miconazole (0.074 mM). Following incubation with moderate doses of miconazole (0.15–0.3 mM), sessile cells of Δefg1(EFG1) were characterized by an intermediate miconazole sensitivity phenotype (Fig. 1a). Based on our expression analysis of EFG1 in *C. albicans* sessile cells (see Discussion), we found the EFG1 expression levels in Δefg1(EFG1) sessile cells to be 18 times lower than those in WT sessile cells, explaining the observed increased miconazole sensitivity of Δefg1(EFG1) sessile cells as compared to WT sessile cells. Following incubation with a high miconazole dose (0.6 mM), sessile cells of Δefg1(EFG1) were as sensitive to miconazole as Δefg1 sessile cells, whereas the WT sessile cells were resistant to miconazole at this dosage. Moreover, in line with the results of Ramage et al. (2002), sessile cells of Δefg1, Δefg1(EFG1) and the WT strain were resistant to 0.6 mM fluconazole (Fig. 1b).

To further confirm these results obtained with the CV quantification method, representing both sessile cells and extracellular matrix, we determined the fraction of viable sessile cells after miconazole treatment by plate counts (Bink et al., 2011). Δefg1 sessile cells showed increased sensitivity to miconazole as compared to WT or reintegrant Δefg1(EFG1) sessile cells. Treatment of Δefg1 sessile cells with 75, 37.5 or 18 μM miconazole resulted in 19.4 ± 10.7, 26.5 ± 24.0 and 46.6 ± 20.1 % survival rates, respectively, whereas treatment of Δefg1(EFG1) or WT sessile cells with 75, 37.5 or 18 μM miconazole resulted in significantly higher survival counts (P<0.05) of 28.0 ± 23.6, 49.1 ± 34.1 or 53.9 ± 29.9 %, respectively, in the case of Δefg1(EFG1) cells, and 76.8 ± 31.3, 96.0 ± 19.5 or 94.0 ± 11.0 %, respectively, in the case of WT cells.

In addition, we analysed the sensitivity of Δefg1 sessile cells to two other commonly used antimycotics, namely AmB and CAS. We found that Δefg1 sessile cells showed increased sensitivity to AmB (BEC50 = 1.5 μM) and CAS (BEC50 = 50 μM) compared to sessile cells of the corresponding isogenic WT strain CAF2 (BEC50 = 12 μM and BEC50 = 90 μM, respectively).

**Intracellular miconazole levels are increased in Δefg1 sessile cells**

Next, we assessed whether miconazole is internalized by the sessile *C. albicans* cells and investigated whether the increased miconazole sensitivity of Δefg1 sessile cells is due to an increased uptake or decreased efflux of miconazole in Δefg1 as compared to WT sessile cells upon miconazole treatment. To this end, we treated 16-h-old Δefg1 and WT *C. albicans* sessile cells with different concentrations of miconazole for 4 h and determined the concentration of miconazole in the adherent cells via HPLC analysis. Treatment of Δefg1 or WT sessile cells with 0.15–0.6 mM miconazole resulted in significantly increased intracellular miconazole levels in Δefg1 sessile cells as compared to WT sessile cells (Fig. 2). These data indicate that the miconazole sensitivity of Δefg1 sessile cells is probably due to increased

**Table 1. Susceptibilities of planktonic (MIC50) and biofilm (BEC50) *C. albicans* mutant cells, affected in cyclic AMP signalling, and corresponding WT strain CAF2 cells to miconazole.**

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC50 (μM)</th>
<th>BEC50 (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caf2</td>
<td>0.0113</td>
<td>&gt;4.8</td>
</tr>
<tr>
<td>Δalc35</td>
<td>0.0068</td>
<td>4.8</td>
</tr>
<tr>
<td>Δras1</td>
<td>0.0203</td>
<td>&gt;4.8</td>
</tr>
<tr>
<td>Δpde1</td>
<td>0.0068</td>
<td>0.1</td>
</tr>
<tr>
<td>Δpde2</td>
<td>0.0068</td>
<td>&gt;4.8</td>
</tr>
<tr>
<td>Δefg1</td>
<td>0.0180</td>
<td>0.8</td>
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</tbody>
</table>
intracellular miconazole accumulation in Δefg1 sessile cells as compared to WT sessile cells.

To determine whether efflux pumps are involved in governing miconazole tolerance of C. albicans biofilms, we investigated the miconazole sensitivity of sessile C. albicans DSY1050 cells, lacking all the major efflux pumps (CDR1, CDR2, MDR1) (Perumal et al., 2007), by using the CV quantification method as described above. We found that the miconazole sensitivity of C. albicans DSY1050 sessile cells was significantly increased compared to sessile cells of the corresponding WT SC5314 cells upon treatment with miconazole ranging from 0.15 to 2.4 mM. Treatment with miconazole concentrations <0.15 mM resulted in comparable sensitivity of C. albicans DSY1050 and the corresponding WT SC5314 cells (data not shown). These data indicate that efflux pumps are an important miconazole tolerance determinant of C. albicans biofilm cells. Whether miconazole efflux is impaired in Δefg1 sessile cells, resulting in increased miconazole accumulation, needs to be investigated further.

Δefg1 sessile cells are sensitive to miconazole in vivo

To assess the increased sensitivity of sessile cells of the Δefg1 mutant to miconazole under in vivo conditions, we used the recently developed subcutaneous biofilm rat model, in which biofilm growth on catheter fragments inoculated with the different C. albicans strains prior to implantation was determined (Ricicova et al., 2010). The numbers of sessile C. albicans cells recovered from the implanted catheters in vivo are given in Fig. 3. There was no difference in the numbers of attached cells of in vivo grown biofilms between Δefg1, Δefg1(EFG1) and WT cells in the absence of miconazole. Miconazole treatment did not result in a reduction in the number of surviving WT cells on the catheters, but miconazole treatment of rats implanted with catheters infected with Δefg1 or Δefg1(EFG1) resulted in a similar and significant (P<0.012 and P<0.007, respectively) reduction in cell numbers compared to vehicle-solution-treated controls for both strains. Note that there was no difference between miconazole sensitivity of sessile cells of Δefg1 and Δefg1(EFG1) in vitro upon treatment with 0.6 mM miconazole or in vivo upon treatment with 200 mg kg⁻¹ miconazole.

DISCUSSION

In this study, we focused primarily on the effect of the antifungal compound miconazole against C. albicans biofilms for several reasons: (i) previous reports showed fungicidal activity of miconazole against young (2–6 h) and mature C. albicans biofilms, in contrast to the fungistatic azole fluconazole (Lamfon et al., 2004; Vandenbosch et al., 2010; Bink et al., 2011), and (ii) the mode of antibiofilm action and resistance mechanisms of
miconazole are fairly well documented; besides inhibiting the ergosterol biosynthesis, miconazole induces ROS in biofilm C. albicans cultures, probably accounting for the observed fungicidal action of miconazole against biofilms (Vandenbosch et al., 2010; Bink et al., 2011). Thus, we used miconazole, which is mainly used to treat topical fungal infections (Kyle & Dahl, 2004), as a model compound to gain information on tolerance mechanisms of C. albicans biofilms against antifungals in general in vitro and in vivo.

Using an in vitro biofilm model, we found that sessile Δefg1 C. albicans mutant cells exhibited increased miconazole sensitivity as compared to WT sessile cells, while their fluconazole resistance was not altered. Moreover, we found that Efg1 is a biofilm-specific miconazole tolerance determinant as planktonic cells of the Δefg1 C. albicans mutant were not characterized by increased miconazole sensitivity as compared to WT cells. Efg1 is a central regulator of numerous cellular processes in C. albicans. Efg1 is required for development of true hyphae and has a dual role as a transcriptional activator and repressor, whose balanced activity is essential for pseudohyphal and hyphal morphogenesis of C. albicans (Stoldt et al., 1997; Tebarth et al., 2003).

The observed fluconazole resistance of Δefg1 sessile C. albicans cells is in line with previous reports, in which it was described that the sessile lifestyle of adherent cells confers antifungal resistance against antifungals including fluconazole. This antifungal resistance of biofilms was, at least, partially efflux-pump-related, regardless of coherent biofilm formation (Mateus et al., 2004), which is apparently affected in Δefg1 C. albicans mutants (Ramage et al., 2002; Watamoto et al., 2009). C. albicans strains affected in Efg1 expression do not produce hyphae or an extracellular matrix, but still form pseudohyphae, resulting in rather sparse monolayers of loosely attached, elongated, rod-like cells (Ramage et al., 2002).

Recently, Prasad et al. (2010) demonstrated a role for Efg1 in mediating tolerance of C. albicans to azoles including fluconazole, ketoconazole and itraconazole and polyenes incuding AmB when grown on solid media. In that study, a Δefg1 C. albicans mutant showed enhanced susceptibility to these drugs on agar, which was independent of multidrug efflux pumps. Moreover, the Δefg1 C. albicans mutant displayed increased membrane fluidity on solid media, leading to enhanced passive diffusion of the drugs (Prasad et al., 2010). We observed a significant increase in intracellular miconazole levels in Δefg1 sessile cells as compared to WT cells following miconazole treatment, explaining, at least in part, the increased miconazole sensitivity of Δefg1 sessile cells. As we found that sessile cells of a C. albicans mutant lacking all major efflux pumps were characterized by increased miconazole sensitivity as compared to sessile cells of the corresponding WT, the presence of efflux pumps is an important determinant governing tolerance of sessile cells to miconazole. Whether the increased miconazole sensitivity and concomitant accumulation of miconazole in Δefg1 sessile cells originates from dysfunctional efflux pumps will be investigated further. In addition, we found increased AmB and CAS sensitivity of Δefg1 sessile cells as compared to WT cells, suggesting that Efg1 is involved in tolerance mechanisms of C. albicans biofilms to other conventional antifungicals as well as miconazole. These findings are in line with the observations of Watamoto et al. (2009), who observed that Δefg1 sessile cells were more sensitive to AmB compared to the isogenic WT, but, in contrast to the current study, were equally resistant to CAS compared to the WT. Moreover, Ramage et al. (2002) found Δefg1 sessile cells to be equally resistant to AmB compared to the WT. Such inter-study variations highlight the differences in susceptibilities of Candida biofilms, which can be strongly influenced by different setups, including the type of device, composition and pH of the medium (Kucharíková et al., 2011) and the quantification/susceptibility methods used (Kuhn et al., 2002b).

We further demonstrated that sessile cells of the EFG1 reintegrant Δefg1(EFG1) showed a reduced EFG1 expression as compared to WT sessile cells, which was consistent with the increased miconazole sensitivity of sessile cells of Δefg1(EFG1) as compared to WT cells. These data link EFG1 expression levels and miconazole sensitivity of C. albicans biofilms. The inability of an EFG1 reintegrant to fully complement a Δefg1 null mutant has been described before (Giussani et al., 2002). As EFG1 has been shown to possess an unusually large 5′ untranslated region (UTR) (1.2 kb), reintroducing EFG1 to the native locus might disrupt proper UTR functioning, leading to lower EFG1 expression levels as compared to WT (Lachke et al., 2003; Srikantha et al., 2000). A similar phenomenon has been described for the transcription factor Czf1 (Vinces et al., 2006).

In the present study, we confirmed the in vitro observations of increased miconazole sensitivity of Δefg1 and reintegrant sessile cells using a rat model for in vivo biofilm analysis. So far, this model has been successfully used for studying the
activity of anidulafungin and fluconazole against in vivo sessile cells (Kuchariková et al., 2010). To the best of our knowledge, this is the first time that miconazole has been tested against in vivo Candida albicans sessile cells. Interestingly, sessile cells of Δefg1 and reintegrant strains were significantly more sensitive to intraepithelially administered miconazole than their isogenic WT. These data confirm the results obtained under in vitro conditions. It is of note that almost 40 % of miconazole-treated Δefg1(EFG1) and 30 % of treated Δefg1 adherent cells constituted less than 2 log₁₀ cells, which is below the diagnostic threshold for catheter-related infections (Mermel et al., 2001).

All the data presented here indicate that Efg1 is implicated in mediating tolerance of Candida albicans sessile cells to various antifungal agents such as miconazole, AmB and CAS. Hence, this transcription factor may be a suitable target for future antifungal therapy.

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