Micafungin activity against Candida albicans with diverse azole resistance phenotypes

Theresa S. Richards1,2, Brian G. Oliver2 and Theodore C. White1,2*

1Department of Global Health, School of Public Health and Community Medicine, University of Washington, Seattle, WA, USA; 2Seattle Biomedical Research Institute, Seattle, WA, USA

Received 20 December 2007; returned 15 February 2008; revised 13 March 2008; accepted 16 March 2008

Objectives: The purpose of this study was to investigate whether mechanisms of azole resistance in Candida albicans contribute to reduced micafungin activity in vitro.

Methods: MICs were determined for a collection of strains with well-characterized mechanisms of azole resistance obtained from systemic, oral and vaginal infections. This collection of strains includes those with resistance-associated phenotypes. All known molecular mechanisms of azole resistance are included in this set of isolates (alone or in combination). Micafungin activity was further investigated for a subset of isolates by agar dilution.

Results: There was no correlation between any of the azole resistance mechanisms or resistance phenotypes and micafungin activity as determined by MIC, even in isolates with cross-resistance to multiple azole drugs. Overexpression of the ABC transporter CDR2 has been suggested to contribute to reduced echinocandin activity in agar dilution studies. By broth microdilution, there was no difference in MIC between the pump overexpressors and the collection as a whole. However, azole-resistant isolates from matched strains exhibited a small increase in their micafungin MICs relative to their susceptible controls. By agar dilution analysis, multiple CDR2-overexpressing strains exhibited reduced growth in the presence of micafungin relative to the laboratory strain SC5314.

Conclusions: Azole resistance mechanisms do not contribute to increased micafungin MIC as determined by broth microdilution. However, within sets of matched isolates, strains overexpressing CDR2 had a slight increase in micafungin MIC. Changes in micafungin susceptibility are associated with CDR2 overexpression in agar dilution tests.

Keywords: echinocandins, antifungals, XTT

Introduction

Candida albicans is the most common opportunistic fungal pathogen of humans and the fourth leading cause of nosocomial infection in the USA. The successful treatment of candidiasis by azoles (particularly fluconazole) has been impaired by the emergence of drug-resistant strains in patients undergoing long-term or prophylactic treatment.

The echinocandins are an emerging class of low-toxicity antifungal drugs effective against many pathogenic fungi. Micafungin is a water soluble, semi-synthetic echinocandin with in vitro and in vivo activity against many fungal species. Caspofungin and anidulafungin, the two other currently approved echinocandins, exhibit paradoxical attenuated activity at high concentrations. While this paradoxical attenuation has been described for micafungin, it is considerably more rare and has not yet been described in C. albicans. As such, micafungin is poised to become a clinically important member of the antifungal armamentarium.

Unlike the azoles, echinocandins act by specific, non-competitive inhibition of the β-(1,3)-d-glucan synthase enzyme complex that catalyses glucan polymers, a major component of fungal cell walls. The reduced activity of echinocandin drugs has been observed in C. albicans strains containing point mutations in FKS1, which encodes a subunit of the glucan synthase complex. There are no orthologues in humans. Owing to the different sites of action of azoles and echinocandins, it is speculated that azole resistance mechanisms will not contribute to reduced activity of micafungin.

Resistance to azole drugs can be achieved through mutation of the drug target gene (ERG11) or increased expression of ERG11 or of genes involved in drug efflux (CDR1, CDR2 and MDR1). A large number of azole-resistant C. albicans strains
have increased expression of CDR1 and CDR2, which encode two ABC transporters, and MDR1, which encodes a major facilitator pump. By up-regulating expression of these genes, alone or in combination, azole levels in the cell are kept low through efflux. All previous studies of micafungin activity against azole-resistant C. albicans have been performed on clinical isolates with uncharacterized mechanisms of azole resistance.\textsuperscript{16–21} This study is the first to evaluate micafungin activity in isolates with known mechanisms of azole resistance. In addition to contributing to azole resistance, overexpression of CDR2 may play a role in reduced activity of echinocandins.\textsuperscript{22–24} Agar dilution studies have suggested a correlation between CDR2 overexpression and reduced caspofungin activity. This is not observed in broth microdilution studies. To investigate this possibility for micafungin and for the strains in this collection, agar dilution was performed on the CDR2 overexpressors and on matched azole-susceptible isolates.

In addition to determining micafungin activity in isolates with well-characterized genotypic mechanisms of azole resistance, micafungin activity was determined for C. albicans with resistance-associated phenotypes including mating-type linked (MTL) resistance, trailing resistance, heterogeneous resistance and inducible resistance.\textsuperscript{25–27} Isolates with MTL resistance have an azole resistance phenotype that correlates with homozygosity at the mating locus possibly associated with alteration in the TAC1 transcription factor.\textsuperscript{2} Residual growth in a high concentration of the drug is a characteristic of a trailing phenotype.\textsuperscript{3} A small number of colonies of heterogeneous-resistant (HET-R) isolates will grow in high drug concentrations, but the cells from these colonies are not intrinsically resistant; even cells growing on high concentrations of the drug will remain azole-susceptible.\textsuperscript{4} In strains with inducible resistance, unstable resistance can be generated through serial passage in the presence of azoles, but is lost in the absence of azoles.\textsuperscript{5}

This study is the first to characterize micafungin activity in a collection of isolates with defined azole resistance mechanisms. Micafungin MICs are determined by turbidity and XTT reduction for this collection. XTT reduction is a quantitative surrogate determination of cell growth.\textsuperscript{28–31} Isolates from multiple biological sites (oral, systemic and vaginal) and encompassing all known mechanisms of azole resistance (alone or in combination) including resistance-associated phenotypes are represented. This collection includes four matched sets of azole-susceptible and -resistant isolates, three from bone marrow transplant patients\textsuperscript{32,33} and one from an HIV patient.\textsuperscript{33–35} In addition to broth-based MIC determinations, to investigate whether CDR2 overexpression plays a role in reduced micafungin activity in agar-based studies, agar dilution was performed on 22 CDR2 overexpressors and on the matched azole-susceptible isolates.

**Materials and methods**

**Organisms and media**

A total of 75 isolates were used in this study,\textsuperscript{14,18,32,36} These isolates have been characterized as C. albicans by their hospital of origin and in this lab by growth on ChromAgar (DRG International, Mountainside, NJ, USA). For long-term storage, cultures were maintained at −80°C in yeast peptone dextrose (YPD; 20 g of dextrose, 20 g of Bacto peptone and 10 g of Difco yeast extract per litre) containing 10% glycerol. Prior to micafungin testing, the isolates were subcultured on YPD at 30°C to ensure growth and purity. Overnight cultures were started from single colonies inoculated into YPD liquid medium and grown at 30°C, 180 rpm.

**Chemicals**

Micafungin (FK-463, Astellas Pharma, Osaka, Japan) was prepared as a 1.6 mg/mL stock solution in water, filtered through a 0.22 μm filter, aliquotted and frozen at −20°C until use. Owing to its poor solubility in water or RPMI, XTT (X6493, Molecular Probes) was prepared as a 1 mg/mL solution in Ringer’s lactate (Phoenix Pharmaceutical, St Joseph, MO, USA) and filtered through a 0.22 μm filter. XTT solution was made immediately prior to use. Menadione (M5625, Sigma-Aldrich, St Louis, MO, USA) was dissolved in acetone as a 10 mM stock solution and stored at room temperature until use.

**Turbidity assay**

MICs were ascertained in accordance with the CLSI reference method M27-A2 using RPMI 1640 medium with shaking to provide increased aeration and reduce clumping that is more problematic with micafungin. Briefly, a micafungin solution was diluted 2-fold in 96-well microdilution plates with a total well volume of 100 μL per well. Wells were inoculated with 100 μL of culture diluted to OD\textsubscript{600} = 0.00005 and incubated in a moist chamber at 35°C with shaking for 24 or 48 h. Final micafungin concentrations ranged from 0.002 to 1.00 mg/L. Endpoints were determined by comparing OD\textsubscript{540} of cells grown in the absence and presence of the drug. The MIC\textsubscript{50} was defined as the lowest concentration of the drug that inhibited 80% of a strain’s growth at 48 h relative to the no-drug control. For each plate of isolates tested, controls were performed using C. albicans FKS1 mutants: S20, S22 or S25 kindly provided by David Perlin, Newark, NJ, USA\textsuperscript{12} and C. albicans ATCC strain 90028.

**XTT reduction assay**

MIC plates were prepared and inoculated as per the turbidity assay described above. After 24 or 48 h of growth, 4 μL of menadione stock solution was added to 8 mL of XTT solution. Fifty microlitres of this solution was added to each well of the 96-well plate. The stock solution was added to 8 mL of XTT solution. Fifty microlitres of this solution was added to each well of the 96-well plate. The plates were incubated for 3 h at 35°C with 180 rpm and then centrifuged to pellet the yeast. Endpoints were determined by comparing the OD\textsubscript{540} of supernatants from cells grown in the absence and presence of the drug. The MIC\textsubscript{50} and MIC\textsubscript{90} were defined as the lowest concentration of the drug that inhibited 50% of XTT reduction at 24 h or 80% of XTT reduction at 48 h, respectively. Control strains were the same as for the turbidity assay.

**Agar dilution assay**

Overnight cultures of cells were diluted to OD\textsubscript{600} of 0.2 in 0.85% NaCl. An aliquot of 2.5 μL of each of three serial 10-fold dilutions was spotted onto an YPD agar plate containing 0.075 mg/L micafungin. Plates were incubated for 24 h at 30°C, the standard temperature for agar dilution. Photographs were taken with a FluoroChem 8900 (Alpha Innotech Corp., San Leandro, CA, USA) and analysed using AlphaEaseFC software. Interpretation of agar dilution is subjective and not quantitative. It is not possible to determine an endpoint, only to compare how well a strain grows in
the absence and presence of the drug when compared with control strains.

Results

Broth microdilution as defined by the CLSI is the clinically accepted standard method for determining MIC. In order to investigate whether any azole resistance mechanism(s) contributes to a reduction in micafungin activity, micafungin MIC was determined by broth microdilution for a collection of 75 isolates with defined azole resistance mechanisms. MIC<sub>80</sub> based on turbidity for a collection of 75 isolates ranged from 0.016 to 0.125 mg/L micafungin (grey bars in Figure 1). Under these conditions, the three FKS1 mutant strains, S20, S22 and S25, formed hyphal mats that could not be resuspended in broth culture. As a result, their MIC<sub>80</sub> could not be determined accurately. MIC<sub>80</sub> at 48 h was determined by turbidity for all isolates excluding the FKS1 mutants. Of the 72 isolates for which MIC<sub>80</sub> could be determined, 25% had MIC<sub>80</sub> ≤ 0.016 mg/L, 83% had MIC<sub>80</sub> ≤ 0.031 mg/L, 97% had MIC<sub>80</sub> ≤ 0.063 mg/L and 100% had MIC<sub>80</sub> ≤ 0.125 mg/L.

When analysis based on turbidity is impractical, surrogate determinations of cell growth must be implemented. C. albicans reduces the tetrazolium salt, XTT, in the presence of an electron-coupling agent (such as menadione) to yield a water soluble, coloured formazan. The intensity of the colour change directly correlates with the number of metabolically active (live) yeast and, as such, is a quantitative surrogate determination of cell growth. 28–31 MIC<sub>80</sub> at 48 h and MIC<sub>50</sub> at 24 h were determined by the CLSI broth microdilution method using XTT reduction as an indicator of cell viability. The MIC<sub>50</sub> at 48 h is the standard for most drugs but, because echinocandins are fungicidal, high levels of cellular debris can confound spectrophotometric analysis. 37 Thus, for echinocandins, determining the MIC<sub>50</sub> at 24 h yields more consistent inter-laboratory results.

MIC<sub>80</sub> as determined by XTT reduction ranged from 0.016 to 0.063 mg/L micafungin excluding FKS1 mutant controls (white bars in Figure 1). The three FKS1 mutant strains, S20, S22 and S25, had MIC<sub>80</sub> > 1.0 mg/L. Cumulatively, of the 72 test isolates, 26% had MIC<sub>80</sub> ≤ 0.016 mg/L, 81% had MIC<sub>50</sub> ≤ 0.031 mg/L and 100% had MIC<sub>80</sub> ≤ 0.063 mg/L. The only strains that do not fall within this range are the FKS1 mutant controls.

Inter-laboratory differences in MIC<sub>80</sub> testing have been reported for the echinocandins. A multicentre study with caspofungin suggested that MIC<sub>50</sub> measured at 24 h yields more consistent results between laboratories. 37 MIC<sub>50</sub> as determined by XTT reduction were evaluated at 24 h and ranged from 0.008 to 0.125 mg/L micafungin excluding FKS1 mutant control strains (black bars in Figure 1). The three control strains, S20, S22 and S25, had MIC<sub>50</sub> > 1.0 mg/L as determined by relative growth, or XTT reduction, at 24 h. Cumulatively, of the 72 test isolates, 19% had MIC<sub>50</sub> ≤ 0.016 mg/L, 88% had MIC<sub>50</sub> ≤ 0.031 mg/L, 97% had MIC<sub>50</sub> ≤ 0.063 mg/L and 100% had MIC<sub>50</sub> ≤ 0.125 mg/L.

For the majority of strains tested, MIC values (MIC<sub>80</sub> determined by turbidity, MIC<sub>80</sub> determined by XTT reduction and MIC<sub>50</sub> determined by XTT reduction) were identical. Compared with turbidity, the MIC<sub>50</sub> determined by XTT reduction for all isolates differed by 2-fold or less, with the majority of isolates having no difference in MIC<sub>80</sub> regardless of testing method. By XTT reduction, 47 isolates had identical MIC<sub>50</sub> and MIC<sub>80</sub>, 26 isolates differed by 2-fold and 2 isolates differed by 4-fold. A 4-fold difference in MIC is the allowable variation in independent tests. Thus, all methods of determining MIC yielded values that were similar, if not the same.

The micafungin MIC<sub>50</sub> as determined by XTT reduction for this collection are summarized in Table 1 and grouped into subcategories based on: biological origin (rows 2–3), azole resistance profiles (rows 4–10), mechanisms of azole resistance (rows 11–16) and resistance-associated phenotypes (rows 17–20). In this collection, there was no correlation between micafungin MIC<sub>50</sub> and the biological origin of the isolate, its azole resistance mechanisms, combination of mechanisms or resistance-associated phenotypes. Even in isolates cross-resistant to multiple azoles, there was no correlation between any azole resistance mechanism and/or resistant phenotype and micafungin activity.

Overexpression of CDR1 or CDR2 may correlate with a slightly higher MIC when compared with other mechanisms of resistance (compare row 11 with the total trend in Table 1). Regardless of the micafungin MIC determination method, the MICs for CDR2 overexpressors were at least 8-fold lower than the FKS1 mutants and similar to the other isolates tested. Likewise, the micafungin MIC<sub>50</sub> for strain DSY1050 that has deletions of MDR1, CDR1 and CDR2 was identical to that for control strain 90028, indicating that the loss of MDR1, CDR1 or CDR2 does not affect micafungin MIC<sub>50</sub> (Table 2).

Previous studies using agar dilution methods suggest that CDR2 may play a role in reduced echinocandin activity. 22,23 To investigate this possibility for micafungin and for the strains in this collection, agar dilution was performed on 22 CDR2 overexpressors (alone or in combination with CDR1, MDR1 or...
Table 1. Micafungin MIC$_{50}$ for azole-resistant isolates by XTT reduction

<table>
<thead>
<tr>
<th>MIC$_{50}$ (mg/L)</th>
<th>≤0.016</th>
<th>0.031</th>
<th>≥0.063</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>14</td>
<td>48</td>
<td>10</td>
<td>72</td>
</tr>
<tr>
<td>Origin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>oral</td>
<td>2</td>
<td>13</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>systemic</td>
<td>6</td>
<td>22</td>
<td>4</td>
<td>32</td>
</tr>
<tr>
<td>Azole resistance</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>susceptible$^a$</td>
<td>3</td>
<td>9</td>
<td>1</td>
<td>13</td>
</tr>
<tr>
<td>resistant$^b$</td>
<td>11</td>
<td>39</td>
<td>9</td>
<td>59</td>
</tr>
<tr>
<td>cross-resistant$^c$</td>
<td>3</td>
<td>16</td>
<td>5</td>
<td>24</td>
</tr>
<tr>
<td>fluconazole</td>
<td>6</td>
<td>22</td>
<td>8</td>
<td>36</td>
</tr>
<tr>
<td>itraconazole</td>
<td>4</td>
<td>16</td>
<td>4</td>
<td>24</td>
</tr>
<tr>
<td>ketoconazole</td>
<td>3</td>
<td>13</td>
<td>5</td>
<td>21</td>
</tr>
<tr>
<td>clotrimazole</td>
<td>2</td>
<td>12</td>
<td>4</td>
<td>18</td>
</tr>
<tr>
<td>Mechanism of resistance</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDR overexpression</td>
<td>1</td>
<td>14</td>
<td>9</td>
<td>24</td>
</tr>
<tr>
<td>MDR1 overexpression</td>
<td>0</td>
<td>5</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>FLU1 overexpression</td>
<td>1</td>
<td>4</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>ERG11 overexpression</td>
<td>0</td>
<td>6</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>ERG11 mutation</td>
<td>0</td>
<td>5</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>unknown$^d$</td>
<td>6</td>
<td>18</td>
<td>0</td>
<td>24</td>
</tr>
<tr>
<td>Resistance-associated phenotype</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MTL</td>
<td>2</td>
<td>9</td>
<td>3</td>
<td>14</td>
</tr>
<tr>
<td>HET-R</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>inducing</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>trailing</td>
<td>3</td>
<td>5</td>
<td>0</td>
<td>8</td>
</tr>
</tbody>
</table>

$^a$Susceptible isolates represent both susceptible and susceptible dose-dependent isolates.
$^b$Isolates resistant to at least one of the azoles listed.
$^c$Isolates resistant to more than one of the azoles listed.
$^d$Isolates that have been characterized, but for which no known resistance-associated gene is overexpressed or mutated.

ERG11), matched azole-susceptible isolates, as well as the common laboratory strain SC5314, and the FKS1 mutant S25. By agar dilution, many CDR2-overexpressing strains were able to grow in the presence of micafungin (Figure 2). When compared with SC5314, 3 isolates had equivalent growth, 7 grew at levels one dilution factor greater, 10 grew better by two dilution factors and 2 had growth equivalent to the FKS1 mutant in the presence of 0.075 mg/L micafungin.

Fluconazole resistance was not predictive of changes in micafungin activity for the strains tested. The three micafungin-resistant FKS1 mutants, control strain 90028 and DSY1050 (CDR2$^{-/}$) were tested for their susceptibility to multiple antifungals (including azole antifungals). By Etest, FKS1 mutant strains had MIC similar to the control strain 90028 for all the antifungals tested: fluconazole, ketoconazole, itraconazole, voriconazole and amphotericin B except for the echinocandins micafungin and caspofungin (Table 2).

For the 22 CDR2-overexpressing isolates tested by agar microdilution, there was no correlation between MICs as determined by the broth microdilution methods (Table 1) and growth on agar (Figure 2). For example, the two isolates that grew as well as the FKS1 mutant by agar dilution have MICs within one dilution factor of SC5314 by all other testing methods. One isolate identified with agar microdilution had MIC similar to the control strain 90028 for all the antifungals tested: fluconazole, ketoconazole, itraconazole, voriconazole and amphotericin B except for the echinocandins micafungin and caspofungin (Table 2).

Figure 2. Agar dilution of CDR2 overexpressors. Serial 10-fold dilutions of exponentially growing cultures were spotted onto YPD plates containing 0.075 mg/L micafungin and incubated at 30°C for 48 h. Strains are: parental azole-susceptible isolate FH1, matched CDR2 overexpressor azole-resistant isolate FH5, azole-resistant CDR2 overexpressor 17, common laboratory strain SC5314 and FKS1 mutant S25. U indicates undiluted.

Table 2. MICs (mg/L) of multiple antifungals for FKS1 mutants, 90028 and DSY1050 (CDR2$^{-/}$)

<table>
<thead>
<tr>
<th>Strains</th>
<th>MFN$^a$</th>
<th>CAS</th>
<th>FLC</th>
<th>KTC</th>
<th>ITC</th>
<th>VRC</th>
<th>AMB</th>
</tr>
</thead>
<tbody>
<tr>
<td>FH1</td>
<td>&gt;1.0</td>
<td>3</td>
<td>0.5</td>
<td>0.006</td>
<td>0.016</td>
<td>0.008</td>
<td>0.047</td>
</tr>
<tr>
<td>FH5</td>
<td>&gt;1.0</td>
<td>3</td>
<td>0.5</td>
<td>0.004</td>
<td>0.016</td>
<td>0.008</td>
<td>0.047</td>
</tr>
<tr>
<td>17</td>
<td>&gt;1.0</td>
<td>4</td>
<td>0.5</td>
<td>0.006</td>
<td>0.016</td>
<td>0.008</td>
<td>0.047</td>
</tr>
<tr>
<td>SC5314</td>
<td>0.016</td>
<td>0.064</td>
<td>0.5</td>
<td>0.008</td>
<td>0.023</td>
<td>0.012</td>
<td>0.094</td>
</tr>
<tr>
<td>S25</td>
<td>0.016</td>
<td>0.023</td>
<td>0.047</td>
<td>&lt;0.002</td>
<td>&lt;0.002</td>
<td>0.002</td>
<td>0.032</td>
</tr>
</tbody>
</table>

MFN, micafungin; CAS, caspofungin; FLC, fluconazole; KTC, ketoconazole; ITC, itraconazole; VRC, voriconazole; AMB, amphotericin B.
$^a$MIC$_{50}$ was determined by broth microdilution. All other MICs were determined by Etest.
## Micafungin activity against *C. albicans*

**Table 3.** Micafungin MICs in matched isolates

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC&lt;sub&gt;S0&lt;/sub&gt;</th>
<th>MIC&lt;sub&gt;S0, XTT&lt;/sub&gt;</th>
<th>MIC&lt;sub&gt;S50&lt;/sub&gt;</th>
<th>0.01</th>
<th>0.03</th>
<th>0.075</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1</td>
<td>0.031</td>
<td>0.031</td>
<td>0.031</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>fluconazole-susceptible</td>
</tr>
<tr>
<td>#17</td>
<td>0.063</td>
<td>0.063</td>
<td>0.063</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>overexpresses MDR1, CDR1 and CDR2</td>
</tr>
<tr>
<td>FH1</td>
<td>0.031</td>
<td>0.031</td>
<td>0.031</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>fluconazole-susceptible</td>
</tr>
<tr>
<td>FH5</td>
<td>0.125</td>
<td>0.063</td>
<td>0.063</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>overexpresses CDR1 and CDR2</td>
</tr>
<tr>
<td>FH8</td>
<td>0.063</td>
<td>0.063</td>
<td>0.063</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>overexpresses CDR1 and CDR2</td>
</tr>
<tr>
<td>FHB1</td>
<td>0.031</td>
<td>0.031</td>
<td>0.016</td>
<td>5</td>
<td>3</td>
<td>2</td>
<td>fluconazole-susceptible</td>
</tr>
<tr>
<td>FHB3</td>
<td>0.031</td>
<td>0.063</td>
<td>0.031</td>
<td>4</td>
<td>3</td>
<td>1</td>
<td>overexpresses CDR1 and CDR2</td>
</tr>
<tr>
<td>90028</td>
<td>0.016</td>
<td>0.016</td>
<td>0.016</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>control laboratory strain</td>
</tr>
<tr>
<td>S25</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>5</td>
<td>5</td>
<td>4</td>
<td>FKS1 mutant</td>
</tr>
</tbody>
</table>

All drug concentrations are expressed in mg/L.

On agar dilution plates, each strain was plated at five different cell concentrations. Numbers represent the number of 10-fold dilutions that were able to grow.

## Discussion

In this collection ofazole-resistant *C. albicans* isolates, micafungin activity is unaffected by any known mechanism of azole resistance, alone or in combination with other mechanisms. Likewise, isolates with resistance-associated phenotypes did not have MICs significantly different from the ATCC strain 90028. This is not surprising given previous findings that azole-resistant isolates have no change in susceptibility to caspofungin.23 This study is the first to determine that no azole resistance mechanism, set of mechanisms or resistant phenotypes contribute to reduced micafungin activity.

While interpretive criteria for micafungin have not been established, strains with point mutations in *FKS1*, the gene encoding a subunit of the glucan synthase complex, had MICs that were >8-fold higher than the highest MIC for the azole-resistant strains. Interestingly, the *FKS1* mutant strains S20, S22 and S25 were all susceptible to azoles and other antifungals by broth microdilution, suggesting that no known mechanism confers cross-resistance to both azoles and micafungin.

The standard method of determining MIC relies on the turbidity of cultures grown in the absence and presence of the drug. The MICs determined by the CLSI are the drug concentrations at which growth of cells exposed to the drug is inhibited 50% and 80%, respectively, when compared with the same isolate grown in the absence of the drug on the same microtitre plate. The MICs at 48 h is the standard for most drugs but, for echinocandins, the MICs at 24 h yields more consistent inter-laboratory results.37,38

Relative growth of *FKS1* mutants was difficult to characterize by turbidity because they formed hyphal mats in broth culture with or without drug and could not be resuspended. XTT reduction was used as a quantitative surrogate determination of cell growth. MICs determined by XTT reduction and turbidity differed by 2-fold or less for all strains tested.

The site of isolation may effect the development of resistance as demonstrated by the paucity of azole-resistant vaginal isolates, despite decades of long- and short-term use of azole drugs to treat vaginal yeast infections.39 In previous studies of azole susceptibility testing, the majority of isolates were collected from the oral cavity. In addition to oral isolates, this collection contains isolates from vaginal and bloodstream infections. There was no correlation between biological site of infection and micafungin MIC (Table 1).

In addition to up-regulation or mutation of resistance-associated genes, resistance to azole drugs can be associated with several drug resistance phenotypes: MTL, HET-R, inducible resistance and trailing resistance (described in the Introduction section and in detail in references 25–27). There was no significant difference in MIC between the ATCC strain 90028 and isolates with inducible resistance, homozygosity at the mating locus or heterogeneous resistance. Inducible resistance can occur after serial passage in the presence of the drug so prolonged passage in the presence of micafungin might lead to transient changes in micafungin MICs though this has not, as yet, been tested. Trailing resistance, characterized by a low level of residual growth in the drug, has been described only for fungistatic drugs. Because micafungin is fungicidal, it is not surprising that there was no significant difference between MIC for the trailing azole-resistant isolates and laboratory control strain 90028.

Of the azole-resistant isolates tested in this study that overexpress a resistance-associated gene, the majority (24/26) express more than one resistance-associated gene in combination. Although multiple azole resistance mechanisms are active in these isolates, there is no indication of reduced micafungin activity by MIC, confirming that micafungin activity is separate from the ergosterol biosynthetic pathway and probably not a substrate for the CDR1, CDR2 and MDR1 pumps.

Twenty-four isolates in this collection are resistant to azole antifungals, but the mechanism of resistance is unknown (i.e. these isolates do not show overexpression or point mutation of resistance-associated genes). Even against these isolates, which may represent entirely new mechanisms of azole resistance, micafungin is effective.

Overexpression of *CDR2* contributes to reduced caspofungin activity in agar dilution studies with a limited number of strains.22,23 In the current study, *CDR2* overexpressors were tested for their susceptibility to micafungin by broth microdilution methods and by agar microdilution. By broth microdilution, *CDR2* overexpressors did not have MICs significantly different from the ATCC strain 90028. In addition, a laboratory-derived
**Richards et al.**

CDR2 deletion strain had an unaltered micafungin MIC. However, in sets of matched isolates, strains that overexpressed CDR2 had MICs that were equal to, or more often higher than, their azole-susceptible partners, suggesting that CDR2 may have a small effect when monitored in accordance with the CLSI. Using agar dilution, 2 of the CDR2 overexpressers had equivalent growth to an FKS1 mutant, 3 had growth equivalent to SC5314 and the remaining 17 strains had intermediate growth, demonstrating that the phenotype is common but variable. In agar dilution studies of matched isolates, CDR2 overexpression in an identical strain background does not correlate with an ability to grow on plates containing higher micafungin concentrations (Table 3).

There are a variety of differences between MIC and agar dilution studies such as differences in oxygen availability, oxygen tension, media, temperature of incubation and time of incubation (for MIC\textsubscript{80}). Additionally, these tests monitor differences in oxygen availability, ability to grow on plates containing higher micafungin concentrations.

**Acknowledgements**

We thank David Perlin (Public Health Research Institute, Newark, NJ, USA), Michael Pfaller (University of Iowa, Iowa City, IA, USA), David Stevens (Stanford University, Stanford, CA, USA), Kieren Marr (Oregon Health & Science University, Portland, OR, USA) and Luis Ostrosky-Zeichner (University of Texas Health Science Center, Houston, TX, USA) for providing us with some of the isolates used in this study.

**Funding**

This research was supported by a Preclinical Research Agreement from Astellas Healthcare, Inc. to T. C. W. and by NIH NIDCR grants R01 DE11367, R01 DE14161 and R01 DE17078. T. S. R. was supported in part by NIH training grant T32 AI07509.

**Transparency declarations**

None to declare.

**References**

Micafungin activity against *C. albicans*


