Comparative Effects of Micafungin, Caspofungin, and Anidulafungin against a Difficult-To-Treat Fungal Opportunistic Pathogen, Candida glabrata

Elisabetta Spreghini, a Fiorenza Orlando, b Maurizio Sanguinetti, c Brunella Posteraro, d Daniele Giannini, d Esther Manso, e and Francesco Barchiesia

Department of Biomedical Sciences and Public Health, Clinic Infectious Disease, Università Politecnica delle Marche, Ancona, Italy; a Experimental Animal Models for Aging Units, Scientific Technological Area N. Masera, I.N.R.C.A.-I.R.R.C.S., Ancona, Italy; b Institute of Microbiology, Università Cattolica del Sacro Cuore, Rome, Italy; c Centro di Gestione Presidenza Medicina e Chirurgia, Università Politecnica delle Marche, Ancona, Italy; d and Laboratory of Microbiology, Azienda Ospedaliero-Universitaria, Ospedali Riuniti Umberto I-Lancisi-Salesi, Ancona, Italy

The aim of this study was to compare the in vitro and in vivo activities of micafungin, caspofungin, and anidulafungin against Candida glabrata. The MICs against 28 clinical isolates showed that the overall susceptibilities to caspofungin and to micafungin were not statistically different in the absence of human serum, whereas the isolates were less susceptible to micafungin than to caspofungin in its presence. Minimum fungicidal concentrations, as well as time-kill experiments, showed that caspofungin was more active than anidulafungin, while micafungin was superior to either caspofungin or anidulafungin without serum; its addition rendered caspofungin and micafungin equally effective. A murine model of systemic candidiasis against a C. glabrata-susceptible isolate was performed to study the effects of all three echinocandins, and kidney burden counts showed that caspofungin, micafungin, and anidulafungin were active starting from 0.25, 1, and 5 mg/kg of body weight/day, respectively. Two echinocandin-resistant strains of C. glabrata were selected: C. glabrata 30, a laboratory strain harboring the mutation Fks2p-P667T, and C. glabrata 51, a clinical isolate harboring the mutation Fks2p-D666G. Micafungin activity was shown to be as effective as or more effective than that of caspofungin or anidulafungin in terms of MICs. In vivo studies against these resistant strains showed that micafungin was active starting from 1 mg/kg/day, while caspofungin was effective only when administrated at higher doses of 5 or 10 mg/kg/day. Although a trend toward colony reduction was observed with the highest doses of anidulafungin, a significant statistical difference was never reached.

Candida glabrata has recently been reported to be the second most common cause of invasive candidiasis, and there are increasing amounts of data showing its important role in determining either superficial or deep-seat infections (4, 18). Systemic infections due to C. glabrata are characterized by a high mortality rate, and they are difficult to treat due to reduced susceptibility of the species to azole drugs, especially to fluconazole (26). According to the published guidelines, amphotericin B can be used to treat infections due to C. glabrata, especially in profoundly immunocompromised hosts (22). Fortunately, the species also appears to be highly susceptible to the echinocandins (i.e., caspofungin, anidulafungin, and micafungin), making these agents valuable alternatives as first-line therapy against this Candida species (22). Interestingly, patients suffering from systemic candidiasis due to C. glabrata showed a trend, although not a significant one, to a better clinical outcome when treated with micafungin (87%) rather than with caspofungin (67%) (23).

The three echinocandin antifungal agents anidulafungin, caspofungin, and micafungin have a unique mechanism of action, inhibiting β-1,3-d-glucan synthase, an enzyme that is necessary for the synthesis of β-1,3-d-glucan polymers, essential components of the cell walls of several fungi (8).

The aim of this study was to compare the effects of all three FDA-approved echinocandins against C. glabrata. In particular, the potential fungicidal activities of these compounds were investigated both in vitro and in a neutropenic murine model of systemic infection due to echinocandin-susceptible and -resistant strains of C. glabrata.

MATERIALS AND METHODS

In vitro studies. (i) Isolates. A total of 30 isolates of C. glabrata were studied: 21 were isolated from blood samples, while the other 7 were recovered from the gastrointestinal, respiratory, and urinary tracts. Each strain represented a unique isolate from a patient. Additionally, two echinocandin-resistant strains were evaluated: C. glabrata 51, a clinical isolate (provided by M. Sanguinetti) that harbors a mutation in FKS2 (A1997G; Fks2p-D666G), as well as a laboratory strain, C. glabrata 30, which harbors another mutation in FKS2 (C1999A; Fks2p-P667T). The latter strain was selected in vitro by plating C. glabrata 11 (caspofungin MIC, 0.03 μg/ml) on 20 μg/ml caspofungin-containing yeast extract-peptone-dextrose (YPD) agar plates. Yeast isolates were identified at the species level by conventional morphological and biochemical methods and were stored at −70°C in 10% glycerol. Before the initiation of the study, yeast
isolates were subcultured on antimicrobial-agent-free medium to ensure viability and purity.

(ii) **Antifungal drugs.** Amphotericin B was used as pure powder (Sigma Chemical, Milan, Italy) for in vitro studies and as a commercial preparation (Fungizone; Bristol-Myers Squibb S.p.A.) for in vivo studies. Anidulafungin (Pfizer, Inc.), caspofungin (Merck & Co., Inc.), and micafungin (Astellas Pharma Inc.) were supplied as pure powders and used for the in vitro and in vivo experiments.

For in vitro studies, solutions of amphotericin B and anidulafungin were prepared in dimethyl sulfoxide (DMSO) (Sigma), while caspofungin and micafungin were prepared in sterile water. Further dilutions were prepared in test medium. For in vivo studies, solutions of amphotericin B, anidulafungin, caspofungin, and micafungin were prepared following the manufacturers’ instructions.

(iii) **Broth dilution.** Antifungal susceptibility testing was performed by the broth microdilution method in accordance with the Clinical and Laboratory Standards Institute (CLSI) document M27-A3 (7). The final concentrations of drugs ranged from 0.002 to 2.0 μg/ml for the susceptible strains and from 0.03 to 16 μg/ml for the resistant strains. Plates were incubated at 35°C for 24 h (48 h for amphotericin B), and readings were performed both visually and spectrophotometrically. Anidulafungin, caspofungin, and micafungin MICs were considered to be the first concentrations of the antifungal agent at which the turbidity in the well was 50% less than that in the control well. Amphotericin B MICs were considered to be the first concentration of the antifungal agent at which there was complete inhibition of growth after 48 h of incubation; the ANID, CAS, and MICA MICs were considered to be the first concentration of the antifungal agent at which there was a visually prominent reduction in growth (approximately 50%) relative to the drug-free growth control after 24 h of incubation.

The MIC was defined as the lowest concentration of antifungal compound yielding no growth.

---

**Table 1: In vitro Susceptibilities of 28 Clinical Isolates of C. glabrata to Amphotericin B, Anidulafungin, Caspofungin, and Micafungin**

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Median MIC (μg/ml)</th>
<th>Median MFC (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AMB</td>
<td>ANID</td>
</tr>
<tr>
<td>C. tropicalis ATCC 750</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. parapsilosis ATCC 22019</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. glabrata</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

---

a AMB, amphotericin B; ANID, anidulafungin; CAS, caspofungin; MICA, micafungin.

b The AMB MIC was defined as the lowest drug concentration at which there was complete inhibition of growth after 48 h of incubation; the ANID, CAS, and MICA MICs were defined as the lowest concentration at which there was a visually prominent reduction in growth (approximately 50%) relative to the drug-free growth control after 24 h of incubation.

c The MFC was defined as the lowest concentration of antifungal compound yielding no growth.

---

The CLSI MIC breakpoints for C. glabrata are based on the use of 1% human serum. However, in this study, studies and as a commercial preparation (Fungizone; Bristol-Myers Squibb S.p.A.) for in vivo studies. Antifungal susceptibility testing was performed as described above and with 50% human serum in the medium. The human serum was prepared from the blood of healthy volunteers.

C. tropicalis ATCC 750 was utilized as an additional strain, and C. parapsilosis ATCC 22019 was included on each day of testing as a CLSI-recommended quality control strain.

(iv) **Killing curves.** Killing experiments were performed against two strains (C. glabrata 11, and C. glabrata 30). Briefly, three to five colonies of
Results

The in vitro susceptibilities of 28 clinical isolates of *C. glabrata* against amphotericin B, anidulafungin, caspofungin, and micafungin, either with or without 50% human serum, are reported in Table 1 and Fig. 1. The geometric mean MICs of anidulafungin, caspofungin, and micafungin were 0.10, 0.04, and 0.02 µg/ml, respectively. In the presence of human serum, the geometric mean MICs of anidulafungin, caspofungin, and micafungin increased to 1.08, 0.32, and 0.62 µg/ml, respectively. Multiple-comparison analysis of MIC values showed that the overall susceptibilities to caspofungin and to micafungin were not statistically different in the absence of human serum, whereas the isolates were less susceptible to micafungin than to caspofungin in its presence (P < 0.05). In general, anidulafungin was the less active echinocandin. The MFCs obtained without serum showed that caspofungin was more active than anidulafungin, while micafungin was superior to either caspofungin or anidulafungin. However, the addition of serum rendered caspofungin and micafungin equally effective. Again, both drugs were more effective than anidulafungin (Fig. 1).

Then, *C. glabrata* 11 was selected to compare the fungicidal activities of all the drugs (Fig. 2). In these experiments, drugs were utilized at concentrations of 0.25×, 1×, 4×, and 32× the MIC with or without human serum. After 24 h of incubation, amphotericin B yielded killing activity at a concentration of 32× the MIC, regardless of the absence or presence of serum. In the absence of serum, micafungin exerted fungicidal activity starting...
from 1× the MIC after 24 h of incubation. At the same time interval, caspofungin was fungicidal at doses of 4× and 32× the MIC, while anidulafungin yielded a fungicidal effect at 32× the MIC. In the presence of serum, anidulafungin, and caspofungin were fungicidal at 4× and 32× MIC, while anidulafungin did not reach fungicidal activity.

C. glabrata 11 was also utilized for in vivo studies. Mice were infected with 1.35 × 10⁸ CFU/mouse, and the drug activities were studied on days 3, 5, and 7 postinfection. The results for kidney tissue burden obtained with this strain are reported in Fig. 3. After 2 days of treatment, caspofungin was active starting at a dose of 1 mg/kg/day, while micafungin was active at doses of 5 and 10 mg/kg/day. On day 5 postinfection, all doses of caspofungin were effective at reducing the burden. Micafungin was active starting at a dose of 1 mg/kg/day. Although, anidulafungin showed a trend in reduction, the agent did not significantly decrease the fungal burden with respect to the control after 2 and 4 days of treatment at any tested doses. On day 7 postinfection, all three echinocandins at 5 and 10 mg/kg/day were effective at reducing the counts against the controls. At 1 mg/kg/day, either caspofungin or micafungin, but not anidulafungin, was active. At the lowest dose tested (0.25 mg/kg/day), only caspofungin was active.

In order to investigate the effects of anidulafungin, caspofungin, and micafungin against echinocandin-resistant isolates of C. glabrata, two additional strains were selected for further in vitro and in vivo studies. C. glabrata 30 is a laboratory strain harboring a mutation in FKS2 (C1999A; Fks2p-P667T), while C. glabrata 51 is a clinical isolate harboring another FKS2 mutation (A1997G; Fks2p-D666G). In vitro susceptibility tests of these two strains are shown in Table 2. Time-kill studies were performed against C. glabrata 30, and the results are shown in Fig. 4. In these experiments, drugs were utilized at concentrations of 0.25×, 1×, 4×, 32×, 64×, 128×, and 256× the MIC with or without serum. Amphotericin B yielded killing activity against this isolate after 24 h of incubation at a concentration of 32× the MIC either with or without serum. Anidulafungin, caspofungin, and micafungin exerted fungicidal activity at 128× the MIC in the absence of serum after 24 h of incubation; in the presence of serum, all three echinocandins were not active against this isolate of C. glabrata.

Both resistant strains of C. glabrata were utilized to compare the efficacies of the echinocandins in vivo, and the results are shown in Fig. 5. Against C. glabrata 30, micafungin was effective at doses of 1, 5, and 10 mg/kg/day and caspofungin was active at 5 and 10 mg/kg/day. Similarly, against C. glabrata 51, micafungin was effective at doses of 1, 5, and 10 mg/kg/day, while caspofungin was active only at a dose of 10 mg/kg/day. Although for both strains anidulafungin at 5 and 10 mg/kg/day showed a trend toward reduction of CFU with respect to the controls, a statistically significant difference was never reached by using the multiple-comparison analyses.

DISCUSSION
Our findings showed that all 28 clinical isolates recovered from patients hospitalized in our department presented anidulafungin, caspofungin, and micafungin MICs within the previously reported ranges for wild-type strains of C. glabrata (3, 5, 24, 25). Also, our results showed that the MICs were within the susceptibility ranges for all three echinocandins, with the exception of two isolates showing an intermediate MIC value for anidulafungin and caspofungin against C. glabrata are ≥0.12 μg/ml for susceptible isolates (S), 0.25 μg/ml for intermediate isolates (I), and ≥0.5 μg/ml for resistant isolates (R), while the CBPs for micafungin are ≥0.06 μg/ml for S isolates, 0.12 μg/ml for I isolates, and ≥0.25 μg/ml for R isolates (24).

It has been reported that echinocandins exert fungicidal activity against yeasts (10, 11). Therefore, we investigated this characteristic by determining either the MFCs or the killing curves. In
In general, our MFCs were within the reported ranges for all three echinocandins (11), with a rank order of activity of micafungin > caspofungin > anidulafungin.

Interestingly, a similar rank order was maintained when the "cidal" activity was investigated by killing experiments. In general, all three echinocandins exerted fungicidal activities against the susceptible isolate of *C. glabrata*. Our results are in agreement with those previously reported for caspofungin by Nagappan et al. (19). These authors assessed the *in vitro* activity of caspofungin against fluconazole-susceptible and -resistant isolates and observed that the drug was fungicidal at concentrations of $1/2$ and $4/2$ g/ml.

A previous study reported that micafungin was fungicidal at concentrations ranging from 4 to 16 times the MIC against *C. glabrata* isolates with MICs ranging from 0.0039 to 0.25 g/ml (9). Similar to this study, our data showed that micafungin exerted fungicidal activity starting from 1 to 32 times the MIC (0.06/2.0 g/ml). In our hands, anidulafungin was fungicidal at concentrations of 32 times the MIC against the susceptible isolate, whereas previous data reported "cidal" activity starting from 4 times the MIC (16, 20). It can be hypothesized that this difference might be due to a slight modification of the experimental procedure (i.e., drug preparation, drug lot, subcultured volumes, "cidal" definition, etc).

It is known that echinocandins bind serum proteins at very high levels (i.e., >99% to human plasma proteins for anidulafungin and approximately 97% to albumin for caspofungin) (15, 21).

**TABLE 2** *In vitro* susceptibility to amphotericin B, anidulafungin, caspofungin, and micafungin of two echinocandin-resistant strains of *C. glabrata*

<table>
<thead>
<tr>
<th><strong>C. glabrata isolate</strong></th>
<th><strong>Median MIC (μg/ml)</strong>&lt;sup&gt;a&lt;/sup&gt;</th>
<th><strong>AMB</strong></th>
<th><strong>ANID</strong></th>
<th><strong>CAS</strong></th>
<th><strong>MICA</strong></th>
<th><strong>AMB</strong></th>
<th><strong>ANID</strong></th>
<th><strong>CAS</strong></th>
<th><strong>MICA</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>RPMI</td>
<td>1.0</td>
<td>0.5</td>
<td>2.0</td>
<td>0.5</td>
<td>1.0</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>51</td>
<td>RPMI + 50% serum</td>
<td>1.0</td>
<td>1.0</td>
<td>2.0</td>
<td>0.25</td>
<td>&gt;16</td>
<td>2.0</td>
<td>2.0</td>
<td>0.5</td>
</tr>
</tbody>
</table>

<sup>a</sup> *C. glabrata* 30 is a laboratory strain selected *in vitro* by plating the isolate *C. glabrata* 11 on 20 μg/ml caspofungin-containing YPD agar plates (Fks2p-P667T); *C. glabrata* 51 is a clinical isolate bearing a mutation in the FKS2 gene (Fks2p-D666G).

<sup>b</sup> AMB, amphotericin B; ANID, anidulafungin; CAS, caspofungin; MICA, micafungin. The MIC was defined as the lowest drug concentration at which there was complete inhibition of growth after 48 h of incubation; the ANID, CAS, and MICA MICs were defined as the lowest concentration at which there was a visually prominent reduction in growth (approximately 50%) relative to the drug-free growth control after 24 h of incubation.
Odabasi et al. (17) evaluated the effects of protein binding on the activities of caspofungin, anidulafungin, and micafungin against *Candida* and *Aspergillus* species. They observed that adding human serum sharply increased the MICs of micafungin and anidulafungin and modestly affected the MIC of caspofungin. However, they also found that the increase in MICs does not appear to be consistent with the rates of protein binding for the three compounds. Therefore, we performed *in vitro* studies by adding 50% human serum to RPMI 1640. Similar to what was observed by others (15, 17, 21), the addition of serum to the medium increased the MICs of all three drugs. We also performed the experiments by adding 50% fetal bovine serum to the medium, and we obtained similar results (data not shown).

In our hands, the ratios of geometric mean MICs (MIC values with/without serum) were 10.8, 8.0, and 31 for anidulafungin, caspofungin, and micafungin, respectively, while the ratios of geometric mean MFCs (MFC values with/without serum) were 0.9 and 17 for caspofungin and micafungin, respectively (the ratio for anidulafungin was not determined because the tested concentrations were too low with respect to the fungicidal range).

In general, our results are in agreement with previous *in vitro* studies showing an increased echinocandin MIC when 50% serum or bovine serum albumin was added to RPMI 1640 (3, 12, 17).

Killing experiments conducted in the presence of serum showed that both caspofungin and micafungin started to be fungicidal at 4 times the MIC and that the addition of serum did not modify the fungicidal activity of caspofungin while it decreased that of micafungin. These results are in line with the higher serum binding levels of micafungin compared to caspofungin (21). Time-kill plots of anidulafungin in the presence of serum never reached the fungicidal effect, and additional studies should be performed by using various drug lots and by increasing the antifungal agent concentration.

Since *in vitro*/*in vivo* correlation is not yet understood, we compared the *in vivo* activities of all three echinocandins in a neutropenic murine model of candidiasis. In our hands, caspofungin proved to be the most active drug (in terms of either time or dose effectiveness) against this susceptible isolate. In fact, caspofungin started to be effective after only 2 days of treatment, while after 6 days, the lowest effective doses were 0.25, 1, and 5 mg/kg/day for caspofungin, micafungin, and anidulafungin, respectively.

Recently, Andes et al. (1, 2) investigated the *in vivo* activities of all three echinocandins against *Candida* spp., including *C. glabrata*, in a neutropenic murine model of disseminated candidiasis. To compare the potencies of antifungal agents, they calculated the 24-h static dose of each echinocandin and the doses required to achieve a 1-log-unit reduction in colony counts (1). They observed that caspofungin required less drug on a mg/kg basis for efficacy against all organisms than did the other two drugs. Actually, the mean static doses were 21.1, 2.47, and 0.33 mg/kg/day for anidulafungin, micafungin, and caspofungin, respectively, while mean doses to achieve 1-log-unit reduction were 39, 5.88, and 1.16 mg/kg/24 h for anidulafungin, micafungin, and caspofungin, respectively. In agreement with our results against *C. glabrata*, the echinocandins showed the following rank order of activity: caspofungin > micafungin > anidulafungin (1).

Our *in vivo* data on anidulafungin are similar to those observed by Gumbo et al. (14). These authors studied the activity of the drug in a neutropenic murine model of disseminated candidiasis due to a fluconazole-susceptible *C. glabrata* isolate. They found that doses of 8 and 10 mg/kg resulted in progressive declines in kidney fungal density, while data for mice that received 2 and 3 mg/kg did not differ significantly from the controls.

When therapeutic options are limited (i.e., azole resistance, renal insufficiency, drug intolerance, etc.), an important clinical question is whether an infection due to a yeast isolate with reduced susceptibility to a given echinocandin might be treated by an increased dose of the same drug or by selecting a new drug belonging to the same family. Recently, Brzankalski et al. (6) showed that caspofungin dose escalation may overcome the *in vitro* resistance of *C. glabrata* and be effective *in vivo* against resistant isolates. Additionally, the same authors suggested that aminocandin, an
investigational echinocandin, has some potential in the treatment of *C. glabrata* infections due to caspofungin-susceptible isolates and that higher doses may be required against isolates with reduced susceptibility to caspofungin (6). Also, Garcia-Effron et al. (13) performed a genetic analysis of *FKS* and *FKS2* genes from 13 echinocandin-resistant *C. glabrata* isolates. They demonstrated that *FKS* mutations influenced the β-1,3-β-glucan synthase kinetics and the *FKS* gene expression and that the mutations were linked to an echinocandin reduced-susceptibility phenotype. In the current study, we investigated the *in vivo* effects of the available echinocandins against two echinocandin-resistant *C. glabrata* isolates, one harboring the mutation Fks2p-P667T and the other the mutation Fks2p-D666G.

In our *in vivo* experiments, a fungicidal effect (i.e., organ sterilization) was never observed regardless of the drug or strain tested, the dosages, or the duration of therapy. In general, all three echinocandins at the active doses showed lower killing rates against the resistant strains than the susceptible strain.

Interestingly, we observed that micafungin retained its efficacy against both fks2 mutant strains, being effective at doses as low as 1 mg/kg/day. In *C. albicans*, Slater et al. (28) investigated the effects of three doses of micafungin (5 h, 29 h, and 53 h postinfection) in a murine model of disseminated candidiasis due to *C. albicans fks1* heterozygous and homozygous mutants at Ser645. They observed that fungicidal activity in animals infected with an FKS1/fks1 heterozygote was reached only with doses as high as 20 mg/kg, while animals infected with the homozygous fks1 mutant failed to respond to any dosage.

In our study, we also demonstrated that caspofungin dose escalation may overcome *in vitro* resistance. In fact, caspofungin was still active at 5 or 10 mg/kg against the two resistant strains. Anidulafungin showed a trend toward reduction of CFU with respect to the controls at 5 and 10 mg/kg/day, but statistically significant differences were never reached. Our results are partially comparable to those reported by Wiederhold et al. (33). They compared caspofungin and anidulafungin *in vitro* and *in vivo* against two clinical isolates of *C. glabrata* with caspofungin MICs of ≥ 1 µg/ml and found that, despite enhanced *in vitro* potency of anidulafungin, treatment with the echinocandin did not result in reductions in tissue burdens greater than those achieved by treatment with caspofungin.

Recently, Wiederhold et al. (32) demonstrated that higher doses of caspofungin (5 and 10 mg/kg) did improve survival against an fks1p-S645P *C. albicans*-resistant isolate, but not against another isolate bearing the mutation fks1p-F641S. The authors concluded that the caspofungin effect against resistant *C. albicans* isolates may be associated with the virulence of the strain. Overall, these results suggest that there might be a linkage between the increased echinocandin MICs, the specific *FKS* mutations, and the potential for a successful clinical outcome.

In conclusion, we compared *in vitro* and *in vivo* effects of anidulafungin, caspofungin, and micafungin against the difficult-to-treat fungal opportunistic pathogen *C. glabrata*. While all three drugs were often fungicidal *in vitro*, they were not able to completely eradicate the infection in this murine neutropenic model of candidiasis. Caspofungin, followed by micafungin, was the most active drug at reducing the kidney burden of mice infected with an echinocandin-susceptible strain. Interestingly, micafungin showed the best *in vivo* antifungal activity against two resistant mutants of *C. glabrata* bearing specific mutations in the FKS2 hot spot region. A limitation of this *in vivo* study is that the mutant isolates showed a low level of resistance to echinocandins, and extrapolations to other mutants with more dominant mutations cannot be made. Further studies with a larger number of strains showing various levels of echinocandin resistance, as well as several schemes for therapies, should be done to better predict treatment success in clinical practice.

**ACKNOWLEDGMENTS**

We thank Pfizer for providing pure anidulafungin, Merck for providing pure caspofungin, and Astellas for providing pure micafungin.

**REFERENCES**
