**In vitro and in vivo effects of echinocandins against Candida parapsilosis sensu stricto, Candida orthopsilosis and Candida metapsilosis**

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**Objectives:** The aim of the present study was to compare, in vitro and in vivo, the effects of caspofungin, micafungin and anidulafungin against Candida parapsilosis complex isolates.

**Methods:** In vitro activities of all three echinocandins were assessed against C. parapsilosis sensu stricto (n = 4), Candida orthopsilosis (n = 4) and Candida metapsilosis (n = 3) using broth microdilution susceptibility testing, minimum fungicidal concentration determination and a killing-curve assay, in the absence and in the presence of 50% human serum. Then, the activities of all drugs were investigated in an immunocompromised murine model of systemic candidiasis. Animals were infected with six isolates (two for each species) and treated with the echinocandins administered at 0.25, 1, 5 and 10 mg/kg/day for six consecutive days. Fungal burdens were assessed in kidney tissues on day 7 post-infection.

**Results:** Geometric mean MICs of caspofungin, micafungin and anidulafungin for C. parapsilosis sensu lato were, respectively, 0.09, 0.14 and 0.20 mg/L without serum, and 0.70, 3.92 and 5.84 mg/L with serum. The fungicidal activity of all three echinocandins was variable; however, the addition of serum reduced the fungicidal effects against these species. In vivo studies showed that caspofungin at 5 and 10 mg/kg/day significantly decreased the kidney burdens with respect to the controls for all isolates, while micafungin was active at 5 and/or 10 mg/kg/day only against C. metapsilosis.

**Conclusions:** Our susceptibility testing showed that caspofungin was the most active echinocandin against all three species. Also, caspofungin resulted in significant therapeutic effects for treatments of experimental systemic infections due to the three species, while micafungin was effective only against C. metapsilosis.

**Keywords:** Candida parapsilosis complex species, anidulafungin, caspofungin, micafungin, candidiasis

**Introduction**

Candida parapsilosis sensu lato is the second/third most common Candida species isolated from bloodstream infections in many regions of the world, particularly in Latin America. It is particularly associated with bloodstream infections in neonates and with catheter-associated candidaemia and intravenous hyperalimentation.

Early work classified C. parapsilosis isolates into three genetically distinct groups (I, II and III). Later, C. parapsilosis groups were replaced by three different related species named Candida parapsilosis sensu stricto, Candida orthopsilosis and Candida metapsilosis. While isolates belonging to these species are generally susceptible to azoles and polyenes, their susceptibilities to the echinocandins are still uncertain. In particular, in vitro susceptibility data on caspofungin, micafungin and anidulafungin indicate that C. parapsilosis sensu lato and Candida guilliermondii are the least susceptible species in the genus Candida. Additionally, it has been reported that caspofungin MICs for both C. orthopsilosis and C. metapsilosis are lower than that for C. parapsilosis sensu stricto. There is a paucity of in vivo data on the effects of each echinocandin compound against these three ‘novel’ species of Candida.
Therefore, the aim of the present study was to compare the effects of caspofungin, micafungin and anidulafungin against clinical isolates of the C. parapsilosis complex. In particular, the potential antifungal activity of these compounds was investigated both in vitro and in neutropenic murine models of systemic infection due to C. parapsilosis sensu stricto, C. orthopsilosis and C. metapsilosis.

### Materials and methods

#### Microorganisms

The following clinical isolates were used in this study: C. parapsilosis sensu stricto (CP #39, CP #42 and CP #A1, blood samples), C. orthopsilosis (CO #134, bronchoaspirate; CO #141, CO #A1 and CO #A2, blood) and C. metapsilosis (CM #88 and CM #A1, blood; CM #450, tissue biopsy). Candida 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. Yeast isolates were identified to species level either by a multilocus sequence typing scheme or by matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry (MALDI–TOF MS; Bruker Daltonics), and 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.

#### Antifungal drugs

Amphotericin B was obtained as a pure powder (Sigma) for in vitro studies and as a commercial preparation (Fungizone; Bristol-Myers Squibb) for in vivo studies. Caspofungin (Merck), micafungin (Astellas Pharma) and anidulafungin (Pfizer) were supplied as pure powders and used for both in vitro and in vivo experiments.

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

#### Broth dilution

Antifungal susceptibility testing was performed by a broth microdilution method in accordance with CLSI document M27-A3. The final concentrations of drugs ranged from 0.03 to 32 mg/L. Plates were incubated at 35°C for 24 h (48 h for amphotericin B), and readings were performed visually. Caspofungin, micafungin and anidulafungin MICs were considered as the lowest concentrations of the antifungal agent at which the turbidity in the well was 50% less than that in the control well. Amphotericin B MIC was considered as the lowest concentration of the antifungal agent at which no turbidity was detectable. After MIC readings, the plates were centrifuged at 2000 rpm, the supernatant was discarded and 50 μL of sterile saline solution was added to each well. The entire volume of sterile saline solution from each well above the MIC for each isolate was withdrawn and plated in duplicate onto a 150 mm Sabouraud dextrose agar (SDA) plate. Then, plates were incubated at 35°C, and minimum fungicidal concentrations (MFCs) were recorded after 48 h. MFC was defined as the lowest concentration of drug yielding no growth (>99% killing). Each test was run in triplicate and repeated on two different days. Antifungal susceptibility testing was performed as described above, with and without 50% human serum in the medium. The human serum was obtained from the blood of healthy volunteers.

### In vivo studies

CD1 female mice weighing 25 g were rendered neutropenic by intraperitoneal (ip) administration of cyclophosphamide (200 mg/kg of body weight/day) on days −4, +1 and +4 post-infection. Yeast organisms were grown on SDA plates +8 h prior to infection and subcultured in yeast extract/peptone/dextrose (YPD) broth overnight at 35°C. Cell density was adjusted using spectrophotometric methods so that it was equivalent to that of a 0.5 McFarland standard (~1×10^8–5×10^8 cfu/mL). A 125 μL aliquot of the fungal suspension was added to 125 μL of either RPMI 1640 medium buffered with MOPS (4×) free of drug or growth medium plus an appropriate amount of drug. Drugs were tested at final concentrations of 2 and 32 mg/L. A 250 μL aliquot of human serum or sterile water was added to each well. Test solutions were placed on a shaker and incubated at 35°C. At timepoints 0, 24 and 48 h following the introduction of the test isolate into the system, 10 μL aliquots were removed from each test solution and a series of 10-fold diluted samples as well as the entire suspension volume from each well were streaked onto SDA plates for colony count determination. Following incubation at 35°C for 48–72 h, the 20 colonies on each plate was determined. Fungicidal activity was considered to be achieved when the number of cfu/mL was <99.9% of that of the initial inoculum. The limit of detection was 2 cfu/mL. All experiments were performed in triplicate and repeated on two different days.

### Statistical analysis

In vitro and in vivo studies were compared among the different groups using the Kruskal–Wallis test, and Dunn’s test for post hoc comparisons between each treatment group and the control group (Prism 5, GraphPad Software). Two-sided p values <0.05 were considered statistically significant.


## Results

In vitro susceptibility results of all drugs against each isolate in this study are reported in Table 1.

Figure 1 shows the geometric mean MICs and MFCs of all three echinocandins for *C. parapsilosis* complex isolates. The geometric mean MICs of caspofungin, micafungin and anidulafungin for *C. parapsilosis sensu lato* were 0.09, 0.14 and 0.20 mg/L, respectively, in the absence of serum, while in its presence the values increased to 0.70, 3.92 and 5.84 mg/L, respectively. Multiple-comparison analysis of MICs showed that caspofungin MICs were statistically different only from those of anidulafungin in the absence of serum, whereas in its presence, caspofungin was more effective than micafungin or anidulafungin (P<0.05). Identical differences among the echinocandins were found within each *Candida* species. The MFC results for *C. parapsilosis sensu lato* showed that all three echinocandins were equally effective without serum, but its addition rendered caspofungin more active than the other two compounds. The statistical analysis of MFC values within each species showed variable fungicidal activity of the three echinocandins; however, addition of serum confirmed the trend of caspofungin being more active than the other two drugs.

Multiple-comparison analysis of the susceptibility results between all three species are reported in Figure 2. In the absence of serum, MICs for *C. metapsilosis* and *C. orthopsilosis* were significantly lower than those for *C. parapsilosis sensu stricto* for all three echinocandins. In the presence of serum, caspofungin showed similar results, while anidulafungin was more active against *C. metapsilosis* than *C. parapsilosis sensu stricto*, and micafungin was equally effective against all three species. In terms of MFCs, *C. metapsilosis* was usually the most susceptible species.

The fungicidal activity of each drug against all isolates was investigated at concentrations of 2 and 32 mg/L, with or without human serum (Table 2). After 48 h of incubation, amphotericin B yielded killing activity even at 2 mg/L, regardless of the absence/presence of serum. In the absence of serum, micafungin and anidulafungin exerted fungicidal activity starting at 2 mg/L after 24 h of incubation, whereas caspofungin was active at the same timepoint and dose against *C. orthopsilosis* and *C. metapsilosis*, but not against *C. parapsilosis sensu stricto*. In the presence of serum, the highest concentration of all three echinocandins exerted fungicidal activity only against *C. metapsilosis*.

Then, we investigated the activity of all drugs against six clinical isolates of *C. parapsilosis* complex in an immunocompromised murine model of systemic candidiasis. The results of kidney tissue burden studies are reported in Figure 3. Amphotericin B at 1 mg/kg/day was effective against all isolates. Caspofungin administered at 5 and 10 mg/kg/day significantly decreased kidney fungal burdens with respect to the control groups against all tested isolates. Micafungin was active at 10 mg/kg/day against both isolates of *C. metapsilosis*, while at 5 mg/kg/day it was effective only against *C. metapsilosis #450*. Although anidulafungin showed a tendency toward burden reduction against some isolates, a statistically significant difference with respect to the controls was never reached.

### Table 1. In vitro susceptibility of *C. parapsilosis* complex isolates to amphotericin B, caspofungin, micafungin and anidulafungin

<table>
<thead>
<tr>
<th>Isolate</th>
<th>RPMI</th>
<th>RPMI plus 50% human serum</th>
<th>Geometric mean MIC (mg/L)</th>
<th>Geometric mean MFC (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AMB</td>
<td>CAS</td>
<td>MCF</td>
<td>ANI</td>
</tr>
<tr>
<td>CT ATCC 750</td>
<td>0.5</td>
<td>0.06</td>
<td>0.06</td>
<td>0.06</td>
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<tr>
<td>CP ATCC 22019</td>
<td>0.4</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
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<tr>
<td>CP #39</td>
<td>0.5</td>
<td>0.125</td>
<td>0.20</td>
<td>0.5</td>
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<tr>
<td>CP #42</td>
<td>0.5</td>
<td>0.25</td>
<td>0.25</td>
<td>0.5</td>
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<tr>
<td>CP #A1</td>
<td>0.25</td>
<td>0.125</td>
<td>0.25</td>
<td>0.25</td>
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<tr>
<td>CO #134</td>
<td>0.5</td>
<td>0.06</td>
<td>0.06</td>
<td>0.125</td>
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<tr>
<td>CO #141</td>
<td>0.5</td>
<td>0.06</td>
<td>0.125</td>
<td>0.125</td>
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<tr>
<td>CO #A1</td>
<td>0.25</td>
<td>0.06</td>
<td>0.1</td>
<td>0.125</td>
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<tr>
<td>CO #A2</td>
<td>0.5</td>
<td>0.125</td>
<td>0.25</td>
<td>0.32</td>
</tr>
<tr>
<td>CM #88</td>
<td>0.5</td>
<td>0.06</td>
<td>0.06</td>
<td>0.08</td>
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<tr>
<td>CM #450</td>
<td>0.5</td>
<td>0.06</td>
<td>0.125</td>
<td>0.1</td>
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<tr>
<td>CM #A1</td>
<td>0.25</td>
<td>0.06</td>
<td>0.06</td>
<td>0.125</td>
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</table>

AMB, amphotericin B; ANI, anidulafungin; CAS, caspofungin; MCF, micafungin.

Each test was run in triplicate and repeated on two different days.

*CM, C. metapsilosis; CO, C. orthopsilosis; CP, C. parapsilosis sensu stricto; CT, C. tropicalis.*

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

The MFC was defined as the lowest concentration of antifungal compound yielding no growth (>99% killing).
Discussion

Our findings showed that all 11 *C. parapsilosis* complex isolates showed caspofungin, micafungin and anidulafungin MICs within the previously reported ranges. According to the revised CLSI clinical MIC breakpoints, none of our isolates was resistant to the echinocandins. Similarly to that reported by Lockart et al., our MICs showed a rank order of activity with...
Table 2. Time–kill assay of amphotericin B, caspofungin, micafungin and anidulafungin against *C. parapsilosis* complex isolates

<table>
<thead>
<tr>
<th>Isolate</th>
<th>AMB (2 mg/L)</th>
<th>CAS (32 mg/L)</th>
<th>AMB (2 mg/L)</th>
<th>CAS (32 mg/L)</th>
<th>AMB (2 mg/L)</th>
<th>CAS (32 mg/L)</th>
<th>AMB (2 mg/L)</th>
<th>CAS (32 mg/L)</th>
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<tbody>
<tr>
<td><em>C. tropicalis</em> ATCC 750</td>
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<tr>
<td>24 h</td>
<td>-1.8 ± 0.1</td>
<td>-6.0 ± 0.1</td>
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<tr>
<td>48 h</td>
<td>-6.0 ± 0.1</td>
<td>-6.0 ± 0.1</td>
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<tr>
<td><em>C. parapsilosis sensu stricto</em> (n=4)</td>
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<tr>
<td>24 h</td>
<td>-3.2 ± 1.3</td>
<td>-6.1 ± 0.4</td>
<td>-6.1 ± 0.1</td>
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<tr>
<td>48 h</td>
<td>-6.1 ± 1.3</td>
<td>-6.1 ± 0.1</td>
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<tr>
<td><em>C. orthopsilosis</em> (n=4)</td>
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<td>24 h</td>
<td>-2.4 ± 0.1</td>
<td>-6.3 ± 0.1</td>
<td>-6.3 ± 0.1</td>
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<td>48 h</td>
<td>-6.3 ± 0.1</td>
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<tr>
<td><em>C. metabasis</em> (n=3)</td>
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<tr>
<td>24 h</td>
<td>-3.9 ± 0.1</td>
<td>-6.4 ± 0.1</td>
<td>-6.4 ± 0.1</td>
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<tr>
<td>48 h</td>
<td>-6.4 ± 0.1</td>
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Each test was run in triplicate and repeated on two different days.

aA total of 12 Candida spp. isolates were tested.

bMean of log10 cfu variation after 24 or 48 h of incubation with amphotericin B (AMB), caspofungin (CAS), micafungin (MCF) or anidulafungin (ANI) compared with that of the initial inoculum (0 h). Data represent the mean result ± standard error for the indicated number of isolates. The bold data represent a >99.9% growth reduction compared with that of the initial inoculum (fungicidal effect). The limit of detection was 2 cfu/mL.
caspofungin > micafungin > anidulafungin against all three species. In agreement with results previously reported by others,2,16 C. metapsilosis and C. orthopsilosis MIC distributions were generally lower than those for C. parapsilosis sensu stricto.

It is known that echinocandins bind serum proteins at very high levels (>99% to human plasma proteins for anidulafungin; ~97% to albumin for caspofungin).12,19 Odabasi et al.20 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 an increase in MIC did not appear to correlate with the level of protein binding for the three compounds. Therefore, we performed in vitro studies with the addition of 50% human serum to RPMI 1640. Similar to observations by the others,12,19,20 the addition of serum to the medium increased the MICs of all three drugs.

In our hands, the ratios of geometric mean MICs (with serum/without serum) for C. parapsilosis sensu stricto, C. orthopsilosis and C. metapsilosis were, respectively, 6.3, 7.8 and 8.3 for caspofungin, 18.0, 34.0 and 38.0 for micafungin, and 26.9, 30.2 and 32.4 for anidulafungin. In general, our results are in agreement with previous in vitro studies showing increased echinocandin MICs when 50% serum or BSA was added to RPMI 1640.20–22

It has been reported that echinocandins exert fungicidal activity against yeasts.23,24 Therefore, we investigated the killing activity of all three echinocandins by both MFC determination and a time–kill methodology. All three echinocandins showed a similar fungicidal activity against C. parapsilosis sensu lato in terms of MFCs, with ranges slightly wider than those previously reported by others.74

Furthermore, killing curves showed that caspofungin, micafungin and anidulafungin were fungicidal against C. orthopsilosis and C. metapsilosis, but not against C. parapsilosis sensu stricto.

Figure 3. Kidney fungal burden of immunocompromised CD1 mice. Animals were infected and treated for six consecutive days with amphotericin B at 1 mg/kg/day (AMB), and caspofungin (CAS), micafungin (MCF) and anidulafungin (ANI) at 0.25, 1, 5 or 10 mg/kg/day. Tissue burden experiments were performed on day 7 post-infection. The bars represent the medians. Asterisks indicate values significantly different from controls (P < 0.05). There were seven or eight mice in each group. C, control.
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even at 32 mg/L. Our results are only partially in agreement with those previously published by others. Cantón et al. showed that the echinocandins were fungicidal against *C. metapsilosis* and *C. orthopsilosis*, but not against *C. parapsilosis sensu stricto*, with the exception of anidulafungin at 8 mg/L after 42 h of incubation. Of note, our strains presented echinocandin MICs lower than those for isolates in the study of Cantón et al., especially that of caspofungin. This might explain the better fungicidal activity that we observed in the present study. However, the addition of serum altered the fungistatic and/or fungicidal activity of the echinocandins against all isolates of *C. parapsilosis sensu stricto* and *C. orthopsilosis*, but not against the three isolates of *C. metapsilosis*. The lack of prior data on killing rates in the presence of serum precluded any kind of comparison with our results, and additional studies should be performed with a larger number of strains.

Since *in vitro* *in vivo* correlation is not yet established, we compared the *in vivo* activity of all three echinocandins in a neutropenic murine model of candidiasis. In our hands, caspofungin was the most active drug against all six tested isolates when administered at 5 and 10 mg/kg/day, while micafungin, at these doses, was effective only against isolates of *C. metapsilosis*. Although, anidulafungin at 10 mg/kg/day often reduced the number of cfu per kidney pair against some isolates, it was never significantly effective against the isolates analysed here. In agreement with our results, Salas et al. tested six strains of *C. parapsilosis sensu stricto* with anidulafungin MICs ranging from 0.12 to 2 mg/L in a murine model of disseminated infection. The animals were treated with different doses of the drug (1, 5 or 10 mg/kg of body weight) and anidulafungin was not able to reduce the tissue burden in those mice infected with the *C. parapsilosis sensu stricto* strains showing MICs higher than 0.12 mg/L.

Recently, Andes et al. investigated the *in vivo* activity of all three echinocandins against *Candida* spp. in a neutropenic murine model of disseminated candidiasis. The authors included in the study 15 *C. parapsilosis* complex isolates, including one *C. orthopsilosis* and one *C. metapsilosis*. To compare the potency of the antifungal agents, they calculated the 24 h static dose (no growth) of each echinocandin and the doses required to achieve a 1 log reduction in colony count. They observed that caspofungin required less drug (mg/kg/24 h) for efficacy against all organisms than did the other two drugs. Actually, the mean static dose and the mean dose to achieve 1 log reduction for caspofungin were 3.56 and 9.84 mg/kg/24 h, respectively, while these in *vivo* pharmacodynamic targets were, respectively, 32.3 and 55.8 mg/kg/24 h for micafungin and 51.0 and 47.0 mg/kg/24 h for anidulafungin. In agreement with our results, caspofungin was active at the highest doses of 5 and 10 mg/kg/day against all *C. parapsilosis* complex isolates studied.

Previously, Garcia-Effron et al. showed that a naturally occurring proline-to-alanine amino acid change in Fks1p in the *C. parapsilosis* group (*C. parapsilosis sensu stricto*, *n* = 3; *C. orthopsilosis*, *n* = 2; *C. metapsilosis*, *n* = 2) accounts for the reduced echinocandin susceptibility compared with other *Candida* species. Also, the authors performed a 1,3-glucan synthase (GS) purification from all strains studied and inhibited the GS complex with several doses of echinocandins to calculate the 50% inhibitory concentrations (IC50s). In line with our *in vivo* results, they observed that IC50s for caspofungin were much lower than those obtained for micafungin and anidulafungin, although they did not analyse the statistical differences between the three sibling species.

Furthermore, we observed that 5 and 10 mg/kg/day micafungin significantly reduced the kidney burden of *C. metapsilosis* isolates with respect to the controls. In agreement with the previous report, micafungin presented a lower IC50 value against one of the two *C. metapsilosis* isolates, and the average IC50 values were lower than those of *C. orthopsilosis* and *C. parapsilosis sensu stricto*. However, the therapeutic effect of micafungin and anidulafungin should be further investigated using a larger number of strains of the *C. parapsilosis* group.

In conclusion, we compared, *in vitro* and *in vivo*, the effects of caspofungin, micafungin and anidulafungin against *C. parapsilosis* complex isolates. Our *in vitro* results showed that caspofungin was the most active echinocandin against all three sibling species. Moreover, dose escalations of caspofungin resulted in significant therapeutic effects for treatments of experimental systemic infections due to *C. parapsilosis sensu stricto*, *C. orthopsilosis* and *C. metapsilosis*, while dose escalations of micafungin were effective only against *C. metapsilosis*. Further studies including a larger number of isolates should be performed to confirm these data and to better predict treatment success in clinical practice.

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Transparency declarations

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Disclaimer

The opinions expressed in this paper are those of the authors and do not represent those of Merck.

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