INTRODUCTION

The echinocandins are the newest antifungal class introduced into clinical practice, and have good efficacy and an excellent safety profile (Andes et al., 2010; Cornely et al., 2011; Mukherjee et al., 2011; Pappas et al., 2009). These drugs are active against triazole-resistant Candida species (e.g. Candida krusei, Candida inconspicua). Because of the recognized increase in fluconazole-resistant Candida infections, echinocandins are currently the first choice for the treatment of invasive Candida infections, including empirical therapy of candidaemias (Pappas et al., 2009; Pfaller & Diekema, 2007; Pfaller et al., 2011).

C. krusei is an important bloodstream pathogen that is responsible for up to 5% of invasive Candida infections (Pfaller & Diekema, 2007; Pfaller et al., 2012; Tortorano et al., 2012). This species is common among neutropenic patients and patients receiving antifungal prophylaxis or corticosteroid therapy. Because C. krusei is resistant to fluconazole, echinocandins are the preferred therapy according to guidelines from the Infectious Diseases Society of America (Chen et al., 2011; Pappas et al., 2009). According to the Prospective Antifungal Therapy Alliance registry, 113 out of 3648 patients suffered from C. krusei candidaemia between 2004 and 2008. C. krusei candidaemia showed the lowest 90-day survival rate (53.6%) among the five most important Candida species. Importantly, 92% of these patients received echinocandin therapy, suggesting that the activity of echinocandins may be suboptimal against this species (Pfaller et al., 2012). Moreover, data on the efficacy of echinocandins against less common species are mostly anecdotal (Pfaller et al., 2011).

Killing rates exerted by caspofungin in 50% serum and its correlation with in vivo efficacy in a neutropenic murine model against Candida krusei and Candida inconspicua

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Killing rates ($K$) of 1–32 $\mu$g ml$^{-1}$ caspofungin were determined in RPMI-1640 and in 50% serum using time–kill methodology against three Candida krusei (MICs of all three isolates 0.25 $\mu$g ml$^{-1}$ in RPMI-1640 and 2 $\mu$g ml$^{-1}$ in serum) and three Candida inconspicua clinical isolates (MIC ranges 0.06–0.12 $\mu$g ml$^{-1}$ in RPMI-1640 and 0.25–0.5 $\mu$g ml$^{-1}$ in serum), against C. krusei ATCC 6258 and against one C. krusei isolate that was resistant to echinocandins (MIC 8 $\mu$g ml$^{-1}$ in RPMI-1640 and 32 $\mu$g ml$^{-1}$ in serum). In RPMI-1640, the highest mean $K$ values were observed at 4 ($1.05$ h$^{-1}$) and 16 ($0.27$ h$^{-1}$) $\mu$g ml$^{-1}$ caspofungin for C. krusei and C. inconspicua clinical isolates, respectively. In 50% serum, mean $K$ value ranges at 1–32 and 4–32 $\mu$g ml$^{-1}$ concentrations for C. inconspicua and C. krusei were $1.12$ to $1.44$ and $0.42$ to $0.57$ h$^{-1}$, respectively. While $K$ values against C. krusei in RPMI-1640 and 50% serum were comparable, serum significantly increased the killing rate against C. inconspicua ($P<0.0003$ for all tested concentrations). In a neutropenic murine model, daily caspofungin at 1, 2, 3, 5 and 15 mg kg$^{-1}$ significantly decreased the fungal tissue burden of C. inconspicua in the kidneys ($P<0.05–0.001$). Against C. krusei, doses of 3, 5 and 15 mg kg$^{-1}$ caspofungin were effective ($P<0.05–0.01$). All effective doses were comparably efficacious for both species. Only the highest 15 mg kg$^{-1}$ caspofungin dose was effective even against the echinocandin-resistant C. krusei isolate. In 50% serum, killing was concentration independent at effective concentrations ($\geq 4$ and $\geq 1$ $\mu$g ml$^{-1}$ for C. krusei and C. inconspicua, respectively), suggesting that the efficacy of dose escalation is questionable. These in vitro results were also supported by the murine model.
Therefore, the aim of our study was to determine the in vitro and in vivo activity of caspofungin (CAS) against C. krusei clinical isolates in order to gain a better understanding of the activity of this drug. Because CAS is a highly protein-bound drug (Andes et al., 2010; Chen et al., 2011; Pfaffer et al., 2011), CAS killing activity was determined in both RPMI-1640 and 50% serum. A less common fluconazole-resistant Candida species, C. inconspicua, was also investigated (Majoros et al., 2003; Pfaffer & Diekema, 2007).

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### METHODS

**Isolates.** All three C. krusei isolates (isolate numbers 4363, 5029 and 27393) and one (isolate number 20114) of three C. inconspicua isolates were isolated from blood samples. The remaining two C. inconspicua isolates were isolated from peritoneal (isolate numbers 4363 and 22027) and wound samples (isolate number 12060). C. krusei type strain ATCC 6258 and one echinocandin-resistant C. krusei isolate (DPL45, FKS F645F/C; courtesy of Professor D. S. Perlin) were also included in the study. C. krusei clinical isolates were identified by APID32C and Matrix-assisted laser desorption/ionization time of flight, while C. inconspicua isolates were identified using molecular biological methods (Majoros et al., 2003).

**Determination of the in vitro activity of CAS.** CAS MICs were determined using the CLSI standard broth macrodilution method (M27-A3) in RPMI-1640, with and without 50% human serum (from a human male, blood type AB; Sigma), at least twice (CLSI, 2008). CAS concentrations ranged between 0.015 and 32 µg ml⁻¹. MICs were read after 24 h using the partial inhibition criterion (at least 50% growth reduction as compared with the growth control) (CLSI, 2008).

In killing studies, CAS activity was measured in RPMI-1640 with and without 50% human serum at 0.5–16 MIC in a final volume of 10 ml using a starting inoculum of approximately 10⁶ cells ml⁻¹ (Foldi et al., 2012a; Garcia-Effron et al., 2011). CAS final concentrations were reduced to one value (Kf=0.3010³/K, Kf=0.3010²/K, Kf=0.3010¹/K, Kf=0.3010⁰/K) for each CAS concentration and each strain (Canton et al., 2009, 2010).

One-way ANOVA with Tukey’s post-testing was used to determine significant differences in killing kinetics among isolates and concentrations in either RPMI-1640 or 50% serum. A t-test (with Welch’s correction, where appropriate) was used for the same CAS concentrations in RPMI-1640 and 50% serum to determine significant differences in killing kinetics between the different media.

### RESULTS

**In vitro experiments**

CAS MICs and killing study results in RPMI-1640 and 50% serum are shown in Table 1. C. krusei clinical isolates were susceptible to CAS according to the revised CLSI breakpoints in RPMI-1640. The C. krusei isolate DPL45 was resistant to CAS (Pfaffer et al., 2011). MIC values were increased four- to eightfold in 50% serum for both C. krusei and C. inconspicua (Table 1).

Mean time–kill data of the three clinical isolates of both species are shown in Fig. 1. Table 2 shows the mean time needed to kill 50, 90, 99 and 99.9% of the starting inoculum of the two species by each CAS concentration in both media, as obtained from the K value of each killing curve regression line (Canton et al., 2009, 2010).
Table 1. Isolates, MICs of CAS and the effect of CAS in time–kill studies in RPMI-1640 and RPMI-1640 supplemented with 50% serum (serum)

<table>
<thead>
<tr>
<th>Isolate</th>
<th>MIC (µg ml⁻¹)</th>
<th>Effect in time–kill studies (µg ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RPMI-1640</td>
<td>Serum</td>
</tr>
<tr>
<td>C. krusei ATCC 6258</td>
<td>0.25</td>
<td>2</td>
</tr>
<tr>
<td>C. krusei 5029</td>
<td>0.25</td>
<td>2</td>
</tr>
<tr>
<td>C. krusei 4363</td>
<td>0.25</td>
<td>2</td>
</tr>
<tr>
<td>C. krusei 27393</td>
<td>0.25</td>
<td>2</td>
</tr>
<tr>
<td>C. krusei DPL45*</td>
<td>8</td>
<td>32</td>
</tr>
<tr>
<td>C. inconspicua 12060</td>
<td>0.125</td>
<td>0.5</td>
</tr>
<tr>
<td>C. inconspicua 22027</td>
<td>0.125</td>
<td>0.5</td>
</tr>
<tr>
<td>C. inconspicua 20114</td>
<td>0.06</td>
<td>0.25</td>
</tr>
</tbody>
</table>

*Echinocandin-resistant isolate (F655F/C).

Fig. 1. Time–kill curves of CAS against the three C. krusei isolates in RPMI-1640 (a) and 50% serum (b), and against the three C. inconspicua isolates in RPMI-1640 (c) and 50% serum (d). Each datum point represents the mean ± SD (error bars) of the three isolates. The broken lines represent the fungicidal limit (3-log decrease).
Table 2. Mean time (h) to achieve 50 (T50), 90 (T90), 99 (T99) and 99.9 % (T99.9) growth reduction from the starting inocula at different CAS concentrations. For groups with multiple isolates (C. krusei clinical isolates and C. inconspicua clinical isolates) SD is also shown.

<table>
<thead>
<tr>
<th></th>
<th>1 μg ml&lt;sup&gt;-1&lt;/sup&gt; CAS</th>
<th>2 μg ml&lt;sup&gt;-1&lt;/sup&gt; CAS</th>
<th>4 μg ml&lt;sup&gt;-1&lt;/sup&gt; CAS</th>
<th>8 μg ml&lt;sup&gt;-1&lt;/sup&gt; CAS</th>
<th>16 μg ml&lt;sup&gt;-1&lt;/sup&gt; CAS</th>
<th>32 μg ml&lt;sup&gt;-1&lt;/sup&gt; CAS</th>
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<tr>
<td></td>
<td>RPMI</td>
<td>Serum</td>
<td>RPMI</td>
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<td>RPMI</td>
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<tr>
<td>C. krusei ATCC 6258</td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T50</td>
<td>0.328</td>
<td>No reduction</td>
<td>0.312</td>
<td>0.346</td>
<td>0.391</td>
<td>0.219</td>
</tr>
<tr>
<td>T90</td>
<td>1.089</td>
<td>No reduction</td>
<td>1.038</td>
<td>1.148</td>
<td>1.299</td>
<td>0.731</td>
</tr>
<tr>
<td>C. krusei (three clinical isolates)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T50</td>
<td>Not done</td>
<td>Not done</td>
<td>Not done</td>
<td>Not done</td>
<td>No reduction</td>
<td>No reduction</td>
</tr>
<tr>
<td>T90</td>
<td>Not done</td>
<td>Not done</td>
<td>Not done</td>
<td>Not done</td>
<td>No reduction</td>
<td>No reduction</td>
</tr>
<tr>
<td>T99</td>
<td>Not done</td>
<td>Not done</td>
<td>Not done</td>
<td>Not done</td>
<td>No reduction</td>
<td>No reduction</td>
</tr>
<tr>
<td>C. inconspicua (three isolates)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>T50</td>
<td>1.27 ± 0.52</td>
<td>0.27 ± 0.02</td>
<td>2.09 ± 1.29</td>
<td>0.23 ± 0.02</td>
<td>1.57 ± 0.19</td>
<td>0.21 ± 0.02</td>
</tr>
<tr>
<td>T90</td>
<td>4.41 ± 1.64</td>
<td>0.89 ± 0.05</td>
<td>4.21 ± 1.37</td>
<td>0.76 ± 0.08</td>
<td>6.21 ± 2.27</td>
<td>0.69 ± 0.06</td>
</tr>
<tr>
<td>T99</td>
<td>8.82 ± 3.28</td>
<td>1.79 ± 0.11</td>
<td>8.43 ± 2.75</td>
<td>1.52 ± 0.16</td>
<td>10.43 ± 1.29</td>
<td>1.39 ± 0.13</td>
</tr>
<tr>
<td>T99.9</td>
<td>13.24 ± 4.92</td>
<td>2.67 ± 0.16</td>
<td>12.64 ± 4.12</td>
<td>2.29 ± 0.24</td>
<td>15.64 ± 1.93</td>
<td>2.09 ± 0.19</td>
</tr>
</tbody>
</table>
Fig. 2. Killing rates of CAS and the corresponding adjusted regression lines (dashed lines) against three C. krusei isolates, C. krusei ATCC 6258, C. krusei DPL45 and three C. inconspicua clinical isolates (means ± SD), each in RPMI-1640 and 50% serum. Positive and negative K values indicate increases and decreases, respectively, in viable cell numbers.
In RPMI-1640 without serum, CAS was fungistatic at \( \geq 0.25 \, \mu g \, ml^{-1} \) (1 x MIC) against C. krusei clinical isolates after 24 h, but regrowth was noticed in the case of isolate 4363 at 0.25–4 \( \mu g \, ml^{-1} \) (1–16 \( \times \) MIC) concentrations. All three isolates were killed at 16 and 32 \( \mu g \, ml^{-1} \) (64–128 \( \times \) MIC) concentrations after 3.75 \( \pm \) 0.71 and 4.64 \( \pm \) 0.33 h (mean \( \pm \) SD), respectively (Table 2). Strain C. krusei ATCC 6258 was killed by \( \geq 1 \, \mu g \, ml^{-1} \) CAS (\( \geq 4 \, MIC \)) after 12–24 h. CAS was fungastic against the resistant strain (C. krusei DPL45) at \( \geq 4 \, \mu g \, ml^{-1} \) in the first 12 h, but regrowth was noticed after 24–48 h. However, 32 \( \mu g \, ml^{-1} \) CAS (4 \( \times \) MIC) proved to be fungicidal (Table 2).

Serum markedly decreased the activity of CAS against C. krusei. In 50 % serum at 1 \( \mu g \, ml^{-1} \) (0.5 \( \times \) MIC), all three C. krusei isolates were inhibited by CAS in the first 4 h, but after 12–24 h killing curves became similar to the control curves (Fig. 1b). CAS at 2–4 \( \mu g \, ml^{-1} \) (1–2 \( \times \) MIC) was fungastic against C. krusei isolates, but regrowth was observed after 24 h. C. krusei clinical isolates reached the fungicidal endpoint after 6.47 \( \pm \) 3.48 and 7.42 \( \pm \) 5.91 h at 16 and 32 \( \mu g \, ml^{-1} \) CAS, respectively (Table 2). Similar killing was noticed in the case of the ATCC 6258 strain. CAS showed a fungastic effect against C. krusei strain DPL45, reaching the maximum c.f.u. decreases at 16–32 \( \mu g \, ml^{-1} \) (\( \leq \) 1g1.82–lg2.44) after 12 h, but regrowth occurred after 24–48 h and the curves became similar to the control as opposed to the data with RPMI-1640 without serum.

CAS at 1 \( \times \) MICs (0.06–0.12 mg l\(^{-1}\)) was fungastic against C. inconspicua in RPMI-1640; all three isolates were killed at \( \geq 0.5 \, \mu g \, ml^{-1} \) CAS (4–8 \( \times \) MIC) at 24 h (Fig. 1c). In 50 % serum, all three C. inconspicua isolates were inhibited at 1 \( \times \) MIC (0.25–0.5 \( \mu g \, ml^{-1} \)) in the first 24 h, but regrowth was observed after 48 h. The fungidical endpoint was reached significantly faster at 1–32 \( \mu g \, ml^{-1} \) CAS in 50 % serum than in RPMI-1640 (Table 2, Fig. 1d).

The relationship between the killing rate and CAS concentrations was linear in both media for both Candida species (Fig. 2). In the case of C. krusei clinical isolates, the highest killing rate was noticed at 4 \( \mu g \) CAS ml\(^{-1}\) in RPMI-1640, which was significantly higher than at 2 and 32 \( \mu g \, ml^{-1} \) (\( P < 0.05 \)). In 50 % serum, the mean K values (range 0.42 to 0.57 h\(^{-1}\)) at effective CAS concentrations (4–32 \( \mu g \, ml^{-1} \)) did not differ significantly (\( P > 0.05 \)) (Fig. 2). The killing rates at 1, 2 and 4 \( \mu g \) CAS ml\(^{-1}\) were significantly higher in RPMI-1640 than in 50 % serum (\( P < 0.001–0.005 \)).

In the case of C. krusei ATCC 6258, at 1 \( \mu g \, ml^{-1} \) (0.5 \( \times \) MIC) growth occurred in 50 % serum, as opposed to RPMI-1640 without serum (Fig. 2). At 2–32 \( \mu g \) CAS ml\(^{-1} \) (1–32 \( \times \) MIC) the K value in RPMI-1640 and 50 % serum ranged from \(-0.46 \) to \(-0.96 \) and from \(-0.87 \) to \(-1.37 \) h\(^{-1}\), respectively. The killing rates at 8 and 16 \( \mu g \, ml^{-1} \) CAS were significantly higher in 50 % serum than in RPMI-1640 (\( P < 0.01 \)).

In the case of the CAS-resistant DPL45 strain, with the exception of 32 \( \mu g \, ml^{-1} \) CAS in RPMI-1640 (the K value at 32 \( \mu g \, ml^{-1} \) was \(-1.12 \) 1 h\(^{-1}\)), killing was observed in neither RPMI-1640 nor 50 % serum (Fig. 2).

Adding 50 % serum significantly increased the CAS killing activity against C. inconspicua at all concentrations tested between 1 and 32 \( \mu g \, ml^{-1} \) (range \(-1.12 \) to \(-1.44 \) h\(^{-1}\), \( P < 0.0003 \) for all tested concentrations; Fig. 2). Different CAS concentrations did produce significantly different K values within the same medium (RPMI-1640 or 50 % serum).

In vivo experiments

All CAS doses decreased the fungal tissue burden by the two C. krusei clinical isolates, but only the 3, 5 and 15 mg kg\(^{-1}\) daily doses were effective (\( P < 0.05–0.001 \); Fig. 3a, b). All effective doses were comparable in efficiency for both isolates (\( P > 0.05 \)). Only the highest CAS dose (15 mg kg\(^{-1}\)) was effective against the C. krusei strain DPL45 (\( P < 0.01 \); Fig. 3c).

In the case of C. inconspicua, all treatment arms decreased the fungal tissue burden in the kidneys at least with two log degrees, which corresponded to a significantly (\( P < 0.05–0.001 \)) lower tissue burden than seen in the untreated control (Fig. 3d, e). Moreover, in the case of C. inconspicua isolate 20114, the fungal tissue burden decreases were higher than four log decreases in mice treated with 5 and 15 mg kg\(^{-1}\) daily doses (Fig. 3e). However, significant differences were not noticed among the effective doses (\( P > 0.05 \)).

DISCUSSION

Echinocandins show concentration-dependent pharmacodynamic activity against susceptible fungi; thus, dose escalation seems to be an option for improving clinical efficacy (Andes et al., 2010; Chen et al., 2011; Mukherjee et al., 2011). In humans, the maximum tolerated daily doses for micafungin and CAS are 900 and 210 mg, respectively (Cornely et al., 2011; Sirohi et al., 2006). However, the benefit of higher dosing of echinocandins for the treatment of invasive Candida infections is still controversial (Betts et al., 2009; Pappas et al., 2007).

The clinical efficacy of dose escalation may be fundamentally influenced by the high degree of protein binding (\( \geq 97 \% \)) of echinocandin antifungals (Andes et al., 2010; Chen et al., 2011; Mukherjee et al., 2011). This is modelled in vitro by adding serum to the test medium; many studies have been performed to compare the activity of echinocandins against Candida and Aspergillus species with and without serum. Both MIC determinations and killing studies have revealed that serum negatively modulates the activity of echinocandins (i.e. MIC values were increased or higher echinocandin concentrations were needed to reach the fungicidal effect) (Földi et al., 2012a; Garcia-Effron et al., 2011; Ishikawa et al., 2009; Odabasi et al., 2007; Paderu et al., 2007; Spreghini et al., 2012; Szilágyi et al., 2012a). However, two studies have reported that serum can enhance the activity of CAS against Candida glabrata and
Aspergillus fumigatus (Chiller et al., 2000; Wiederhold et al., 2007). Moreover, serum-based MIC determination has been reported to identify clinical isolates with acquired mechanisms of resistance against echinocandins more efficiently than the standard method (Garcia-Effron et al., 2011). Unfortunately, serum-based susceptibility methods have not yet been standardized (Nasar et al., 2013).

In 2009, Cantón and colleagues determined the CAS killing rate against C. krusei clinical isolates (MIC range...
0.5–2 µg ml\(^{-1}\)) using RPMI-1640 as test medium. The mean time to reach the fungicidal endpoint at 8 and 32 µg ml\(^{-1}\) was 29.2 ± 6.0 and 21.4 ± 5.9 h, respectively, as calculated from the killing rate (K) (Cantón et al., 2009). The corresponding data in our study were different (4.2 ± 48 h at 8 µg ml\(^{-1}\) CAS and 4.64 ± 0.33 h at 32 µg ml\(^{-1}\) CAS); however, CAS MIC values against C. krusei were lower (0.25 µg ml\(^{-1}\)) for all three isolates) in our study. This discrepancy confirms that even slightly higher MIC values may lead to a marked decrease in or loss of killing activity, which is reflected in the newer breakpoints (the breakpoint of ≤ 2 µg ml\(^{-1}\) was changed to ≤ 0.25 µg ml\(^{-1}\)) (Pfaller et al., 2011). These data also show that killing kinetics can differ even among isolates with similar or identical MICs.

Adding 50 % serum to the medium increased MIC values four- to eightfold against both species. The killing rate at effective CAS concentrations (4–32 µg ml\(^{-1}\)) was concentration independent against C. krusei clinical isolates, since no statistically significant differences were noted among the different CAS concentrations tested. This means that when a critical effective concentration is achieved, further concentration increases hardly influence the results.

Although the reference strain behaved slightly differently, this can be attributed to long in vitro storage or strain variation (the reference strain was isolated from sputum as opposed to the clinical isolates, which originated from blood).

These results were also confirmed in the neutropenic murine model. Both in vitro and in vivo results suggest that a very high dose of CAS (and probably of other echinocandins as well) does not offer much therapeutic benefit against C. krusei. However, dose escalation of CAS to 15 mg kg\(^{-1}\) day\(^{-1}\) proved to be effective against the heterozygous C. krusei mutant strain in vivo. Similar results were obtained by Wiederhold et al. (2011), who found 10 mg kg\(^{-1}\) CAS to be efficacious against one of two echinocandin-resistant C. albicans isolates in reducing the fungal tissue burden. However, these results remain anecdotal and their clinical utility is unknown.

Interestingly, C. inconspicua behaved differently. Although the MICs were increased slightly by 50 % serum, CAS at 1–32 µg ml\(^{-1}\) concentrations significantly increased killing rates in 50 % serum against C. inconspicua when compared with RPMI-1640. Similar results have been reported for C. glabrata and A. fumigatus by Chiller et al. (2000) and Wiederhold et al. (2007); however, Chiller et al. (2000) used only 5 % serum in their experiments. The killing activity of CAS against C. inconspicua, similarly to C. krusei, was concentration independent, but started at concentrations as low as 1 µg ml\(^{-1}\) CAS. In the neutropenic mouse model, 1 mg kg\(^{-1}\) CAS day\(^{-1}\) proved to be effective, significantly decreasing the fungal burden in the kidneys and suggesting that CAS is a highly effective therapy against this rare fluconazole-resistant opportunistic yeast.

This is the first study in which the killing rate produced by an echinocandin drug has been compared head to head in RPMI-1640 and 50 % serum against Candida species. CAS MICs were increased by 50 % serum, and there were no differences between concentrations with significant efficacy as opposed to RPMI-1640 without serum. The killing activity of CAS against C. inconspicua, but not against C. krusei, was significantly higher in the presence of 50 % serum. For these reasons, the clinical utility of echinocandin dose escalation against the two species tested is questionable. Further preclinical and clinical studies are needed to confirm that dose escalation offers any benefit against Candida species when compared with the presently used standard echinocandin therapeutic strategy.

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