Dose escalation studies with caspofungin against Candida glabrata

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Echinocandins are recommended as first-line agents against invasive fungal infections caused by Candida glabrata, which still carry a high mortality rate. Dose escalation of echinocandins has been suggested to improve the clinical outcome against C. glabrata. To address this possibility, we performed in vitro and in vivo experiments with caspofungin against four WT C. glabrata clinical isolates, a drug-susceptible ATCC 90030 reference strain and two echinocandin-resistant strains with known FKS mutations. MIC values for the clinical isolates in RPMI 1640 were ≤0.03 mg l⁻¹ but increased to 0.125–0.25 mg l⁻¹ in RPMI 1640 + 50 % serum. In RPMI 1640 + 50 % serum, the replication of C. glabrata was weaker than in RPMI 1640. Caspofungin in RPMI 1640 at 1 and 4 mg l⁻¹ showed a fungicidal effect within 7 h against three of the four clinical isolates but was only fungistatic at 16 and 32 mg l⁻¹ (paradoxically decreased killing activity). In RPMI 1640 + 50 % serum, caspofungin at ≥1 mg l⁻¹ was rapidly fungicidal (within 3.31 h) against three of the four isolates. In a profoundly neutropenic murine model, all caspofungin doses (1, 2, 3, 5 and 20 mg kg⁻¹ daily) decreased the fungal tissue burdens significantly (P<0.05–0.001) without statistical differences between doses, but the mean fungal tissue burdens never fell below 10⁶ cells (g tissue)⁻¹. The echinocandin-resistant strains were highly virulent in animal models and all doses were ineffective. These results confirm the clinical experience that caspofungin dose escalation does not improve efficacy.

INTRODUCTION

Invasive Candida glabrata infections have increased worldwide, including in Latin America where the dominant non-albicans species are Candida parapsilosis and Candida tropicalis (Colombo et al., 2014; Lortholary et al., 2014; Pfaffer et al., 2012). As C. glabrata clinical isolates often show resistance to fluconazole, echinocandins are recommended as the primary treatment for invasive C. glabrata infections (Alexander et al., 2013; Betts et al., 2009; Colombo et al., 2014; Farmakiotis et al., 2014, 2015; Li et al., 2015; Lortholary et al., 2014; Pappas et al., 2007, 2009; Pfaffer et al., 2012). The mortality rate due to C. glabrata is high in intensive care units where 50–69% mortality rates have been reported (Farmakiotis et al., 2014, 2015; Lortholary et al., 2014). This unacceptably high mortality rate is especially alarming because echinocandin usage, as first-line therapeutic agents, has doubled or tripled in intensive care units (Colombo et al., 2014; Farmakiotis et al., 2014, 2015; Lortholary et al., 2014).

Echinocandins show concentration-dependent activity (Cmax) against Candida spp. and thus higher doses may hypothetically produce better outcomes (Andes et al., 2010; Chen et al., 2011; Howard et al., 2011; Perlin, 2014; Wiederhold et al., 2011). Based on findings with the echinocandin-susceptible C. glabrata ATCC 2001 strain, Howard et al. (2011) suggested that echinocandin dose escalation combined with early treatment [5 h post-infection (p.i.)] may be a viable strategy to achieve a greater antifungal effect in a neutropenic murine model of invasive C. glabrata infection. In order to extend our knowledge about the value of echinocandin dose escalation against C. glabrata, we determined caspofungin killing activity in RPMI 1640 and RPMI 1640 plus 50% serum against echinocandin-sensitive and -resistant clinical isolates. In addition, in a profoundly neutropenic murine model, we performed caspofungin dose escalation studies against the same clinical isolates.

METHODS

Strains. We studied four randomly selected WT C. glabrata bloodstream isolates cultured in 2013–2014 (Table 1); all four were incident isolates (i.e. isolated prior to antifungal administration). Identification was performed as described previously (Kovács et al., 2014a).
The goodness of fit for each isolate was assessed by et al.

Analysis of Caspofungin activity was determined in RPMI 1640 and 50% serum. Without the drug, the mean time to achieve a 1 log increase in c.f.u. in RPMI 1640 and 50% serum. Without the drug, the mean time to achieve a 1 log increase in c.f.u. compared with the starting inoculum was calculated from the growth rate values (k). Calculation of the growth rate corresponded to calculation of the killing rate; negative killing rates indicated growth (see above).

One-way ANOVA with Tukey’s post-test 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 caspofungin concentrations in RPMI 1640 and 50% serum to determine significant differences in killing kinetics in the different media (Kovács et al., 2014a, b).

In vivo studies. Groups of seven to eight BALB/c male mice (23–25 g) were given 150 mg cyclophosphamide kg\(^{-1}\) 4 days before infection, 100 mg cyclophosphamide kg\(^{-1}\) 1 day before infection, 100 mg cyclophosphamide kg\(^{-1}\) 2 days p.i. and 100 mg cyclophosphamide kg\(^{-1}\) 5 days p.i. (Berényi et al., 2014; Foldi et al., 2012b; Kovács et al., 2014a). The animals were maintained in accordance with the Guidelines for the Care and Use of Laboratory Animals; experiments were approved by the Animal Care Committee of the University of Debrecen (permission no. 12/2014). In vivo experiments were performed using all four clinical isolates and the DPL27 and DPL245 strains. Mice were challenged intravenously through the lateral tail vein; the infectious doses were 6.6 \times 10^2–8.4 \times 10^2 c.f.u. per mouse in a 0.2 ml volume. This inoculum size has been shown to produce consistently high fungal tissue burden without mortality (Berényi et al., 2014; Foldi et al., 2012b; Kovács et al., 2014a). For the echinocandin-resistant strains, these doses led to 100% mortality within 5 days; thus, the experiments with the resistant strains were repeated with a lower inoculum of 2.5 \times 10^2 c.f.u. per mouse, which did not cause mortality during the experiments.

A 5-day intraperitoneal treatment with daily 1, 2, 3, 5 and 20 mg caspofungin kg\(^{-1}\) (Cancidas, commercial preparation, obtained from the university pharmacy) was started at 24 h p.i. to allow the fungi to establish tissue infection (Berényi et al., 2014; Foldi et al., 2012b; Kovács et al., 2014a, Spreghini et al., 2012; Wiederhold et al., 2007). This dosing strategy was based on previous pharmacokinetic studies (Andes et al., 2010; Cornelly et al., 2011; Flattery et al., 2011; Mignola et al., 2011; Stone et al., 2002), on previous results from our study group (Berényi et al., 2014; Foldi et al., 2012b; Kovács et al., 2014a) and on the findings of Howard et al. (2011) At the beginning of therapy (day 1), the fungal kidney burden was determined after dissection of four untreated mice for each isolate (day 1 control burden) (Berényi et al., 2014). On day 6 p.i., all mice were sacrificed; both kidneys were removed, weighed and homogenized aseptically. Aliquots of 0.1 ml of the undiluted and diluted (1:10) homogenates were plated onto SDA plates and incubated at 35 °C. C.f.u. numbers were determined after 48 h. The lower limit of detection was 50 c.f.u. (g tissue)\(^{-1}\). The kidney burden was analysed using the Kruskal–Wallis test with Dunn’s post-test for multiple comparisons (Berényi et al., 2014; Foldi et al., 2012b; Kovács et al., 2014a, Spreghini et al., 2012; Wiederhold et al., 2007).

RESULTS

In vitro studies

MIC results in RPMI 1640 and 50 % serum. The MIC values are shown in Table 1. The clinical isolates and the ATCC type strain were susceptible to caspofungin in RPMI 1640 (Pfaffer et al., 2011). Paradoxical growth was not noted in MIC tests, and in 50 % serum, the MICs increased by four- to eightfold. The DPL27 and DPL245 strains were resistant to caspofungin regardless of medium.

Time–kill results in RPMI 1640 and 50 % serum. All isolates grew significantly better in RPMI 1640 than in 50 % serum. Without the drug, the mean time to achieve a 1 log increase in c.f.u. in RPMI 1640 and 50% serum was 8.14 and 11.17 h, respectively, for the clinical isolates and 8.57–8.95 and 14.76–14.86 h, respectively, for the resistant isolates. Representative time–kill plots are shown in Fig. 1.

### Table 1. MICs of caspofungin in RPMI 1640 and 50 % serum

<table>
<thead>
<tr>
<th>C. glabrata isolate</th>
<th>MICs (mg l(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RPMI 1640</td>
</tr>
<tr>
<td>ATCC 90030</td>
<td>≤0.03</td>
</tr>
<tr>
<td>11900</td>
<td>≤0.03</td>
</tr>
<tr>
<td>9098</td>
<td>≤0.03</td>
</tr>
<tr>
<td>18910</td>
<td>≤0.03</td>
</tr>
<tr>
<td>15242</td>
<td>≤0.03</td>
</tr>
<tr>
<td>DPL27</td>
<td>8</td>
</tr>
<tr>
<td>DPL245</td>
<td>&gt;32</td>
</tr>
</tbody>
</table>

Two echinocandin-resistant strains of clinical origin, DPL27 (S663P mutation) and DPL245 (S629P mutation), and the type strain ATCC 90030 were also tested.

Susceptibility testing. MICs of caspofungin (Sigma) in RPMI 1640 and in RPMI 1640 supplemented with 50 % serum (hereafter referred to as 50% serum) (human serum from a male, type AB; Sigma) were determined simultaneously using the standard CLSI (2008) broth macrodilution method (Foldi et al., 2012a, b; Garcia-Effron et al., 2011; Szilágyi et al., 2012). The caspofungin final concentration ranged between 0.03 and 32 mg l\(^{-1}\). MICs were read visually after 24 h using the partial inhibition criterion (CLSI, 2008; Pfaffer et al., 2011).

Time–kill studies. Caspofungin activity was determined in RPMI 1640 and 50% human serum at 0.25, 1, 4, 8, 16 and 32 mg l\(^{-1}\) in a final volume of 10 ml (Foldi et al., 2012a, b; Garcia-Effron et al., 2011; Kovács et al., 2014a, b; Nasar et al., 2013; Szilágyi et al., 2012). The starting inocula were 3.5 \times 10^5–5 \times 10^5 c.f.u. ml\(^{-1}\). For the echinocandin-resistant isolates, only the concentrations 8, 16 and 32 mg l\(^{-1}\) were used. Aliquots of 100 µl were removed at 0, 4, 8, 12, 24 and 48 h, serially diluted 10-fold, plated (4 \times 30 µl) onto a single Sabouraud dextrose agar (SDA) plate and incubated at 35 °C for 48 h (Foldi et al., 2012a, b; Kovács et al., 2014a, b; Szilágyi et al., 2012). All experiments were performed in both media twice.

Analysis of in vitro data. The fungidal activity of caspofungin was defined as a 99.9% or higher reduction in viable cell count compared with the starting inoculum (Cantón et al., 2009, 2010; Gil-Alonso et al., 2015). Killing kinetics at the tested concentrations were analysed in both media (RPMI and RPMI 1640 plus 50% serum), as described previously (Cantón et al., 2009, 2010; Foldi et al., 2012a, b; Gil-Alonso et al., 2015; Kovács et al., 2014a, b). An exponential equation was fitted to the mean data at each time point: N\(_t\) = N\(_0\) \times e\(^{-kt}\), where N\(_t\) is the number of viable yeasts at time t, N\(_0\) is the number of viable yeasts in the initial inoculum, k is the killing rate and t is the incubation time. The six time points on each killing curve were reduced to one value (k). Positive k values indicated killing and negative k values indicated growth. The goodness of fit for each isolate was assessed by the r\(^2\) value (r\(^2\) > 0.8). The mean times to achieve the fungicidal end point (T\(_w\) = t/k) were calculated from the k values for each isolate and concentrations in both media as described previously (Cantón et al., 2009, 2010; Gil-Alonso et al., 2015; Kovács et al., 2014a, b).

For control strains, the time to achieve a 1 log increase in c.f.u. compared with the starting inoculum was calculated from the growth rate values (T = 1/k). Calculation of the growth rate corresponded to calculation of the killing rate; negative killing rates indicated growth (see above).
In RPMI 1640, caspofungin killing activity was better at 1 and 4 mg l\(^{-1}\) than at 16 and 32 mg l\(^{-1}\) against all clinical isolates (Table 2). The mean times to achieve 99.9% growth reduction from the starting inoculum were shorter than 7 h for isolates 9098, 18910 and 15242 and for the ATCC type strain (Table 3). Caspofungin at 16 and

![Time–kill curves of caspofungin against C. glabrata isolate 9098 in RPMI 1640 (a) and 50 % serum (d), isolate 15242 in RPMI 1640 (b) and 50 % serum (e) and isolate DPL27 in RPMI 1640 (c) and 50 % serum (f). The dotted lines represents the fungicidal limit (3 log decrease).](image)
Table 2. Maximum changes in log c.f.u. ml-superscript-1-comparable with the starting inoculum in time–kill studies in RPMI 1640 and 50 % serum

<table>
<thead>
<tr>
<th>C. glabrata isolate</th>
<th>Medium</th>
<th>Maximum log decrease in c.f.u. in time–kill experiments at the indicated caspofungin concentration (mg l-superscript-1-)</th>
<th>0.25</th>
<th>1</th>
<th>4</th>
<th>8</th>
<th>16</th>
<th>32</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC 90030</td>
<td>RPMI 1640</td>
<td>−0.66</td>
<td>−3.60</td>
<td>−3.60</td>
<td>−3.60</td>
<td>−3.60</td>
<td>−3.60</td>
<td>−3.60</td>
</tr>
<tr>
<td></td>
<td>50 % serum</td>
<td>−1.70</td>
<td>−3.60</td>
<td>−3.60</td>
<td>−3.60</td>
<td>−3.60</td>
<td>−3.60</td>
<td>−3.60</td>
</tr>
<tr>
<td>11900</td>
<td>RPMI 1640</td>
<td>−1.67*</td>
<td>−2.00*</td>
<td>−2.78</td>
<td>−2.10</td>
<td>−1.08</td>
<td>−0.54</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50 % serum</td>
<td>−0.01*</td>
<td>−4.00</td>
<td>−4.00</td>
<td>−4.00</td>
<td>−4.00</td>
<td>−4.00</td>
<td>−4.00</td>
</tr>
<tr>
<td>9098</td>
<td>RPMI 1640</td>
<td>−1.78</td>
<td>−3.35</td>
<td>−3.15</td>
<td>−2.62</td>
<td>−1.02</td>
<td>−0.85</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50 % serum</td>
<td>−0.42*</td>
<td>−3.85</td>
<td>−3.85</td>
<td>−3.85</td>
<td>−3.85</td>
<td>−3.85</td>
<td>−3.85</td>
</tr>
<tr>
<td>15242</td>
<td>RPMI 1640</td>
<td>−3.08</td>
<td>−4.00</td>
<td>−4.00</td>
<td>−4.00</td>
<td>−3.18</td>
<td>−2.39</td>
<td>−1.71</td>
</tr>
<tr>
<td></td>
<td>50 % serum</td>
<td>−0.78</td>
<td>−1.76</td>
<td>−2.31</td>
<td>−2.31</td>
<td>−2.42</td>
<td>−2.67</td>
<td></td>
</tr>
<tr>
<td>DPL27</td>
<td>RPMI 1640</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>−0.48*</td>
<td>−0.46</td>
<td>−0.86</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50 % serum</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>−0.20*</td>
<td>−1.08*</td>
<td>−1.90</td>
<td></td>
</tr>
<tr>
<td>DPL245</td>
<td>RPMI 1640</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>Growth</td>
<td>Growth</td>
<td>−0.08*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50 % serum</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>Growth</td>
<td>Growth</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ND, Not done. *Regrowth occurred.

32 mg l-superscript-1-was fungistatic against strain DPL27, but for strain DPL245, a weak and transient fungistic effect was observed at 32 mg l-superscript-1-during the first 8 h in the killing study.

In 50 % serum, the time to reach a fungicidal effect was significantly shorter (≤ 3.32 h) than in RPMI 1640 (Table 3). The killing activity of caspofungin in 50 % serum was better at 32 mg l-superscript-1-than in RPMI 1640, even against the resistant strain DPL27 (Fig. 1, Table 2).

Killing rates in RPMI 1640. The caspofungin killing rates for clinical isolates 11900, 18910, 9098 and the ATCC strain were paradoxically higher at 0.25 and 1 mg l-superscript-1-than at 16 and 32 mg l-superscript-1 (P<0.05–0.001) (Fig. 2). In the case of isolate 15242, the k values were significantly higher at 0.25, 1 and 32 mg l-superscript-1-than at 4, 8 and 16 mg l-superscript-1, but did not differ significantly from each other. Numerically, the highest k values were observed with isolate 9098 at 1 and 4 mg l-superscript-1 (1.058 h-superscript-1-at both concentrations). The lowest k value was noted with isolate 11900 at 32 mg l-superscript-1 (0.177 h-superscript-1).

For the two echinocandin-resistant strains, the k values were always negative (indicating growth) with the exception of the DPL27 isolate at 32 mg l-superscript-1 (0.204 h-superscript-1).

Killing rates in 50 % serum. The killing rate values at 0.25 mg l-superscript-1-showed a wide range; the k value was negative (indicating growth) for isolate 11900 (−0.059 h-superscript-1), but positive (indicating killing) for isolates 9098 (+0.916 h-superscript-1), 18910 (+0.006 h-superscript-1) and 15242 (+0.265 h-superscript-1) and for the ATCC strain (+0.031 h-superscript-1).

The killing rates at 4–32 mg l-superscript-1-for isolates 11900 (range 1.079–1.152 h-superscript-1), 18910 (range 1.061–1.509 h-superscript-1), 9098 (range 1.511–1.536 h-superscript-1) and 15242 (0.480–0.607 h-superscript-1) were concentration independent (P>0.05 for all comparisons) (Fig. 2). For the ATCC strain, the highest k value was detected at 16 mg l-superscript-1 (1.426 h-superscript-1); the k values at 1, 4, 8 and 32 mg l-superscript-1 (range 1.022–1.085 h-superscript-1) did not differ significantly. The k values for the DPL245 strain were uniformly.

Table 3. Time to reach 99.9 % growth reduction (T99,9) from the starting inoculum at different caspofungin concentrations (mg l-superscript-1-) in RPMI 1640 and 50 % serum

<table>
<thead>
<tr>
<th>C. glabrata isolate</th>
<th>Medium</th>
<th>Time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.25</td>
</tr>
<tr>
<td>ATCC 90030</td>
<td>RPMI 1640</td>
<td>3.07</td>
</tr>
<tr>
<td></td>
<td>50 % serum</td>
<td>NA</td>
</tr>
<tr>
<td>11900</td>
<td>RPMI 1640</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>50 % serum</td>
<td>NA</td>
</tr>
<tr>
<td>9098</td>
<td>RPMI 1640</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>50 % serum</td>
<td>NA</td>
</tr>
<tr>
<td>18910</td>
<td>RPMI 1640</td>
<td>3.31</td>
</tr>
<tr>
<td></td>
<td>50 % serum</td>
<td>NA</td>
</tr>
<tr>
<td>15242</td>
<td>RPMI 1640</td>
<td>5.67</td>
</tr>
<tr>
<td></td>
<td>50 % serum</td>
<td>NA</td>
</tr>
<tr>
<td>DPL27</td>
<td>RPMI 1640</td>
<td>NA</td>
</tr>
<tr>
<td>DPL245</td>
<td>RPMI 1640</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>50 % serum</td>
<td>NA</td>
</tr>
</tbody>
</table>

NA, Not achieved; ND, not determined.
Fig. 2. Killing rates of caspofungin and the corresponding adjusted regression lines (dashed lines) against four C. glabrata clinical isolates, the ATCC 90030 reference strain and two echinocandin-resistant strains (DPL27 and DPL245) in RPMI 1640 and 50 % serum. Positive and negative k values indicate a decrease and increase, respectively, in viable cell numbers.
negative at 8–32 mg l⁻¹, but for the DPL27 isolate, the k values at 16 and 32 mg l⁻¹ were 0.065 and 0.354 h⁻¹, respectively.

**Comparison of killing kinetics at the same caspofungin concentrations in RPMI 1640 and 50% serum.** The k values at 0.25 mg l⁻¹ with the exception of isolate 9098 were higher in RPMI 1640 than in 50% serum for all isolates (P<0.05–0.001). The killing activity of caspofungin in 50% serum was significantly higher at 4–32 mg l⁻¹ than in RPMI 1640 for isolates 9098, 1891 and, 11900 and for the ATCC strain (P<0.05–0.001) (Fig. 2, Table 2). For isolate 15242 at 4, 8 and 16 mg l⁻¹, the k values were higher in 50% serum than in RPMI 1640 (P<0.05–0.01), but at 32 mg l⁻¹, a significant difference was not observed.

For DPL27, the killing rates at 16 and 32 mg l⁻¹ (0.07 and 0.36 h⁻¹, respectively) were significantly higher in 50% serum than in RPMI 1640 (Fig. 2).

**In vivo experiments**

The initial inoculum used (7 × 10⁷ cells ml⁻¹) killed all mice before the pre-determined end point; dead animals showed a consistent increase in mean fungal burden over time [6.8 × 10⁷ and 7.2 × 10⁶ cells (g kidney tissue)⁻¹] at day 2 p.i. to 4.3 × 10⁷ and 6.8 × 10⁶ cells (g tissue)⁻¹ at day 5 p.i. for DPL27 and DPL245, respectively. In contrast, all mice infected with susceptible isolates survived, with a mean fungal burden of between 10⁷ and 10⁶ cells (g kidney tissue)⁻¹ at day 6 p.i. in untreated controls. For this reason, resistant isolates were tested at a lower infectious dose (see above) to harmonize the end point for all tested isolates.

At the beginning of therapy (day 1), the mean fungal tissue burden ranges for clinical isolates and resistance strains (DPL27 and DPL245) in untreated control mice were 4.4 × 10⁶–6.7 × 10⁶ and 9.7 × 10⁶–1.4 × 10⁷ cells g⁻¹ per mouse, respectively. All mice in all groups survived. All tested isolates, except isolate 11900, grew <1 mean log unit in untreated control mice as determined by day 6.

All caspofungin doses decreased the fungal tissue burden significantly for all tested clinical isolates compared with the controls (Fig. 3). However, the mean fungal tissue burdens never fell below 10⁵ cells g⁻¹, and statistically significant differences between the five treatment regimens were not observed. Paradoxically decreased activity of caspofungin by 20 mg kg⁻¹ was also not observed.

Against the echinocandin-resistant strains, caspofungin was ineffective regardless of the elevated doses. However, for isolate DPL27 but not for DPL245, growth inhibition was observed compared with the control at day 1 with all three treatment regimens.

**DISCUSSION**

In agreement with other studies (Andes et al., 2010; Fernández-Silva et al., 2014; Gil-Alonso et al., 2015; Lepak et al., 2012; Pfaffer et al., 2011; Spreghini et al., 2012; Szilágyi et al., 2012), caspofungin showed excellent *in vitro* activity against WT clinical isolates as well as against the ATCC 90030 type strain. However, in RPMI 1640, caspofungin killing activity decreased at 16–32 mg l⁻¹ when compared with the killing at 0.25–1 mg l⁻¹ (Table 3). This paradoxically decreased activity of caspofungin at higher caspofungin concentrations is part of the stress response of cells characterized by increased chitin content (Ben-Ami et al., 2011; Perlin, 2014). Theoretically, this stress adaptation allows the development of a more profound resistance mechanism over time for surviving fungi (Perlin, 2014). In accordance with previous experiments (Szilágyi et al., 2012), adding 50% serum eliminated the paradoxically decreased activity of caspofungin at 16–32 mg l⁻¹ noted in RPMI 1640 for isolates 11900, 9098 and 18910, as indicated by the high k values (Fig. 2); caspofungin killing activity was concentration independent between 4 and 32 mg l⁻¹. Based on this excellent killing activity of caspofungin in 50% serum, we expected a similar high degree of fungal elimination from the kidneys by all treatment regimens applied.

In accordance with other authors (Andes et al., 2010; Fernández-Silva et al., 2014; Lepak et al., 2012; Spreghini et al., 2012; Wiederhold et al., 2007), WT *C. glabrata* clinical isolates were highly susceptible *in vivo* to caspofungin even at 1 mg kg⁻¹ in mice (corresponding to a 35 mg daily dose in humans) (Cornely et al., 2011; Flattery et al., 2011; Migoya et al., 2011). Moreover, as observed previously with *Candida albicans*, *Candida krusei* and *Candida inconspicua*, increasing the drug dose further did not yield better efficacy (Berényi et al., 2014; Kovács et al., 2014a), i.e. all treatment arms were unable to reduce the mean fungal burden below 10⁵ c.f.u. (g kidney tissue)⁻¹ (Fig. 3). This suggests that, even at higher doses, only suppression of fungal growth can be achieved and thus higher doses do not have a therapeutic benefit compared with lower doses.

Despite the *in vitro* fungicidal effects, the *in vivo* activity of caspofungin was only fungistatic, in line with previous results (Spreghini et al., 2012; Wiederhold et al., 2007). Although immediate or early caspofungin treatment may lead to decreased fungal tissue burdens, treatment started 24 h after challenge did not. The delay in treatment allowed the establishment of high tissue fungal burden in the deeply neutropenic host, modelling the most probable clinical situation; in some cases, the unfavourable therapeutic response may be the result of delayed antifungal treatment (Colombo et al., 2014; Pappas et al., 2009; Pfaffer et al., 2012). The importance of early treatment against disseminated candidiasis was elegantly demonstrated by MacCallum & Odds (2004) for *C. albicans* in an immunocompetent murine model and by Howard et al. (2011) and Lepak et al. (2012) for *C. glabrata* in neutropenic murine models. These
Fig. 3. Kidney tissue burden of the profoundly neutropenic BALB/c mice infected intravenously with *C. glabrata* isolate 11900 (a), isolate 18910 (b), DPL27 (c), isolate 9098 (d), isolate 15242 (e) and DPL245 (f). Daily intraperitoneal caspofungin (CAS) (1, 2, 3, 5 and 20 mg kg$^{-1}$) treatment was started at 24 h p.i. Fungal kidney tissue burden was determined at the beginning of the therapy on day 1 to set the day 1 control burden (control 1) and at the end of experiments on day 6 (control 6). Bars represent medians. The level of statistical significance compared with the control population on day 6 is indicated: *$P<0.05$; **$P<0.01$; ***$P<0.001$. 

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observations based on animal models were confirmed in clinical situations (Farmakiotis et al., 2015).

Moreover, the randomly selected bloodstream isolates used in the present study showed weak replicating ability, as demonstrated both in 50% serum and in vivo. The decreased growth of our WT C. glabrata isolates in 50% serum is consistent with a previous report (Garcia-Effron et al., 2011), and the low in vivo growth rate suggests a relatively low virulence. Considering this, the extremely weak elimination capacity of the treatments was even more notable. A potential explanation may lie in the activity of echinocandins; i.e. the slower growth in tissues may lead to slower cell wall synthesis, which may, in turn, weaken the in vivo killing activity of caspofungin, similarly to what is seen with antibacterial agents inhibiting cell wall synthesis (Chen et al., 2011; Perlin, 2014). Certainly, other factors (e.g. neutropenia, poor drug penetration into the inflamed tissues) influence therapeuetic outcome (Farmakiotis et al., 2014; Pfaffer et al., 2012). Whether the relatively low in vivo fitness found in this study is common among C. glabrata bloodstream isolates, as well as its clinical importance, remain to be determined in further studies.

Although resistance of Candida spp. to echinocandins is low worldwide (Perlin, 2014), secondary resistance to caspofungin at some medical centres is more substantial (Alexander et al., 2013; Beyda et al., 2014; Pappas et al., 2007; Perlin, 2014; Pfaffer et al., 2012; Shields et al., 2013). In a tertiary-care cancer centre in the USA, caspofungin resistance rates of C. glabrata among patients suffering from cancer (Farmakiotis et al., 2014) and acute leukaemia (Wang et al., 2015) were 10.3% (15 out of 146 isolates) and 58.3% (7 out of 12 isolates), respectively. Both studies reported a strong correlation between caspofungin resistance and increased 28-day all-cause mortality rates, suggesting that caspofungin-resistant isolates are more virulent than susceptible ones (Farmakiotis et al., 2014; Wang et al., 2015). These alarming clinical observations were confirmed by Borghi et al. (2014) in their animal model using sequential C. glabrata clinical isolates with and without the S663P mutation. The impact of echinocandin resistance on the virulence of the fungi seems to be species dependent, however, as the FKS1 mutant C. albicans was demonstrated to exhibit reduced fitness and virulence in animal models (Ben-Ami et al., 2011). Our preliminary experiments revealed that the virulence of the caspofungin-resistant isolates was higher than that of WT clinical isolates. Moreover, resistant isolates produced slightly higher fungal burdens at day 6 p.i. at markedly lower challenge doses. However, the susceptible and the resistant isolates were not isogenic.

This study confirmed the clinical experience that the efficacy of echinocandins is not enhanced by higher doses against echinocandin-susceptible clinical isolates (Betts et al., 2009; Pappas et al., 2007), and even higher doses are inefficient against isolates with prominent FKS-mediated resistance (Berényi et al., 2014; Fernández-Silva et al., 2014; Lepak et al., 2012; Spreghini et al., 2012). As C. glabrata rapidly develops resistance to echinocandins, even during short treatment periods (Alexander et al., 2013; Beyda et al., 2014; Lepak et al., 2012; Perlin, 2014; Shields et al., 2013), rapid detection of the most common FKS mutations would best be achieved by FKS sequencing in C. glabrata isolates, as published for C. albicans (Dudiuk et al., 2015). As mutations in the FKS genes are not equal in terms of the level of resistance provided (Beyda et al., 2014; Fernández-Silva et al., 2014; Lepak et al., 2012), and non-FKS-related mechanisms may also emerge over time (Perlin, 2014), MIC testing in RPMI 1640 with or without 50% serum is still useful to detect isolates with decreased susceptibility (Farmakiotis et al., 2014; Perlin, 2014; Wang et al., 2015). Finally, the fitness and virulence of echinocandin-susceptible and -resistant isogenic isolates should be compared in future studies to obtain relevant data on the relationship between echinocandin resistance and virulence.

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REFERENCES


