

1 **Killing rates of caspofungin in 50 percent serum correlate with caspofungin efficacy**  
2 **against *Candida albicans* in a neutropenic murine model**

3

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16

17 **Abstract**

18 Previous studies suggested that caspofungin dose escalation against *Candida* species is  
19 more beneficial than currently used lower daily doses. Thus, we determined *in vitro* and *in*  
20 *vivo* activity of caspofungin against six wild-type *C. albicans* clinical isolates, the ATCC  
21 10231 strain and an echinocandin resistant strain. MIC ranges of clinical isolates in RPMI-  
22 1640 with and without 50% serum were 0.125-0.25 and 0.015-0.06 mg/L, respectively.  
23 Two and three isolates showed paradoxical growth in MIC and time-kill tests, respectively,  
24 in RPMI-1640 but not in 50% serum. Caspofungin killing rate (*k*) in RPMI-1640 at 1 mg/L  
25 was higher than at 16 and 32 mg/L for all isolates ( $p < 0.001$ ). Killing rates for five of six  
26 isolates were concentration independent between 1-32 mg/L in 50% serum ( $p > 0.05$  for all  
27 comparisons), but for one isolate *k* value at 32 mg/L was significantly lower than at 1-16  
28 mg/L. Although *k* values at 1-32 mg/L showed a great variability in 50% serum (the lowest  
29 and highest *k* value ranges were 0.085-0.109 and 0.882-0.985 1/h, respectively), daily 3, 5  
30 and 15 mg/kg caspofungin was effective in a neutropenic murine model against all isolates,  
31 without significant differences between the effective doses. This study confirms that  
32 paradoxical growth does not affect the *in vivo* efficacy of caspofungin. We demonstrated  
33 that dose escalation did not increase the efficacy of caspofungin against *C. albicans* either  
34 *in vitro* or *in vivo*. These results are in concordance with the clinical experience that  
35 efficacy of echinocandins does not increase at larger doses.

36

37 **1. Introduction**

38 Echinocandins show low MIC values and concentration-dependent fungicidal or  
39 fungistatic activity against the majority of *Candida* species in test media RPMI-1640 and  
40 antibiotic medium 3 [1-9]. Moreover, echinocandins proved to be highly effective in the  
41 treatment of invasive infections caused by *Candida* species both in animal models and in  
42 clinical situations [1-4, 10, 11].

43 Echinocandins are increasingly used as first line agents in candidemia and other types of  
44 deep-seated life-threatening infections [1-4, 10-12]. Unfortunately, mortality rate due to  
45 invasive *Candida* infections is still increasing (mean mortality rate around 40% at present)  
46 [10-13]. Although the basic illness is recognized as the most important risk factor of death  
47 among severely ill patients, appropriate antifungal treatment is a key factor for clinical  
48 success [2, 4, 10-13], therefore optimizing the echinocandin therapy is an urgent challenge.

49 As echinocandins are highly protein-bound antifungals ( $\geq 97.5\%$ ) serum or tissue proteins  
50 may alter their activity [1-5]. Both decreased and increased *in vitro* activities of  
51 echinocandins against *Candida* and *Aspergillus* species were demonstrated when the test  
52 medium was supplemented with 5-50% serum in the broth microdilution (BMD) MIC tests  
53 [14-18]. Time kill studies in 50% serum revealed decreased echinocandins activity against  
54 *Candida* species [19, 20].

55 In our previous studies we have compared the caspofungin killing rate in RPMI-1640 and  
56 RPMI-1640 plus 50% serum against *C. albicans*, *C. krusei* and *C. inconspicua* isolates [19,  
57 20]. We noticed increased MIC values in RPMI-1640 plus 50% serum and concentration  
58 independent killing activity of caspofungin at 1-32 mg/L against the three species.  
59 Moreover, when the same isolates were tested with various caspofungin doses in a  
60 neutropenic murine model in cases of *C. krusei* and *C. inconspicua* no differences were

61 found between effective doses in decreasing the fungal kidney tissue burden [19]. Such  
62 comparisons have not yet been performed against the most frequently isolated *C. albicans*.  
63 The aim of this study was to test the *in vivo* activity of caspofungin in a neutropenic  
64 murine model against *C. albicans* clinical isolates. In order to compare *in vivo* activity to *in*  
65 *vitro* efficacy, caspofungin killing rate in RPMI-1640 and RPMI-1640 plus 50% serum  
66 were determined.

67

## 68 **2. Materials and methods**

### 69 *2.1. Strains*

70 We studied six randomly selected *C. albicans* bloodstream strains isolated in 2014 in our  
71 Laboratory (Table 1). All isolates were unique and first isolates (isolated prior to antifungal  
72 administration). Isolates were identified with conventional methods and MALDI Biotyper  
73 (Bruker, Bremen, Germany) [20]. In the preliminary experiments two isolates (10781 and  
74 34350) showed paradoxical growth (PG) in the BMD test. PG is defined as growth at  
75 supra-MICs [6, 7]. Two echinocandin resistant strains, DPL18 (F641S) and DPL20  
76 (F645P) and three ATCC type strains (*C. albicans* ATCC 10231, *C. krusei* ATCC 6258  
77 and *C. parapsilosis* ATCC 22019) were also evaluated in the study [20, 21].

### 78 *2.2 Susceptibility testing*

79 Two test media were used, RPMI-1640 as recommended by CLSI (referred to as RPMI)  
80 and RPMI-1640 supplemented with 50% human serum from a human male, type AB,  
81 Sigma, Budapest, Hungary (referred to as 50% serum) [16-20, 22, 23]. Caspofungin  
82 (Sigma, Budapest, Hungary) MICs in RPMI and in 50% serum were determined  
83 simultaneously using the standard CLSI BMD method. Caspofungin final concentration

84 ranges were 0.015-32 mg/L. MICs were determined after 24 h according to the partial  
85 inhibition criterion [16-20, 23].

86 MIC values were also determined using Etest method (AB Biodisk, Sweden) [24]. Etest  
87 was carried out using freshly prepared RPMI 1640 agar supplemented with 2% glucose  
88 with and without 50% human serum. Etest was carried out according to the instructions of  
89 the manufacturer and the results were read after 24 hours [3, 23, 24].

90

### 91 2.3. *Time-kill studies*

92 As results for ATCC 10231 and the echinocandin resistant strain DPL20 were reported in  
93 previous studies [20], in killing studies we tested only the six *C. albicans* clinical isolates  
94 in the present study. Caspofungin activity was determined in RPMI-1640 with and without  
95 50% human serum at 0.25, 1, 4, 8, 16 and 32 mg/L concentrations using a starting  
96 inoculum of  $\sim 10^5$  cells/ml in a final volume of 10 ml [16-20]. Aliquots of 100  $\mu$ l were  
97 removed after 0, 4, 8, 12, 24 and 48 hours of incubation, tenfold serial dilutions were  
98 prepared, and samples of dilutions (4x30  $\mu$ l) were plated onto a single Sabouraud dextrose  
99 agar (SDA) and incubated at 35 °C for 48 hours [16-20]. All experiments were performed  
100 in both media with two repetitions.

101

### 102 2.4. *Analysis of in vitro data*

103 Caspofungin activity was defined as fungicidal when at least 99.9% reduction in viable cell  
104 count was observed as compared to the starting inoculums [6-9, 16-20, 22].

105 Killing kinetics at the tested concentrations were analysed in both media (RPMI and 50%  
106 serum), as described previously [8, 9, 19, 20]. Briefly, an exponential equation was fitted  
107 to the mean data at each time point:  $N_t = N_0 \times e^{-kt}$ , where  $N_t$  is the number of viable yeasts

108 at time  $t$ ,  $N_0$  is the number of viable yeasts in the initial inoculum,  $k$  is the killing rate, and  $t$   
109 is the incubation time. Thus killing rate represents the overall killing capability of the drug,  
110 taking into account of killing at each tested concentration. Negative  $k$  values indicate  
111 growth and positive  $k$  values indicate killing. The goodness of fit for each isolate was  
112 assessed by the  $r^2$  value ( $r^2 > \pm 0.8$ ) [19, 20]. The mean times to achieve the fungicidal  
113 endpoint ( $T_{99.9} = 3/k$ ) were calculated from the  $k$  values for each isolate and concentrations  
114 in both media [8, 9, 19].

115 Killing kinetics among isolates was compared using one-way ANOVA with Tukey's post-  
116 testing in either RPMI-1640 or 50% serum. The effect of the same drug concentration in  
117 RPMI-1640 and 50% serum was analysed using  $T$  test (with Welch's correction, where  
118 appropriate) [19, 20].

### 119 2.5. *In vivo* studies

120 Groups of seven to eight female BALB/c mice (20-22 g) were immunosuppressed with  
121 four doses of cyclophosphamide, i.e. 4 days before infection (150 mg/kg), 1 day before  
122 infection (100 mg/kg), 2 days postinfection (100 mg/kg) and 5 days postinfection (100  
123 mg/kg) [16, 19, 20]. The Guidelines for the Care and Use of Laboratory Animals was  
124 strictly followed during maintenance of the animals; experiments were approved by the  
125 Animal Care Committee of the University of Debrecen (permission no. 12/2008). All six  
126 clinical isolates as well as the ATCC 10231 and the DPL20 strains were tested in *in vivo*  
127 model. Mice were inoculated intravenously through the lateral tail vein with an infectious  
128 dose of  $7.5 \times 10^4$  CFU/mouse. Confirmation of inoculum density was performed using  
129 plating serial dilutions onto SDA plates [16, 19, 21].

130 Five-day intraperitoneal treatment with daily 1, 2, 3, 5 and 15 mg/kg caspofungin  
131 (Cancidas, commercial preparation) was started after 24 hours [16, 19, 21]. This dosing  
132 strategy was based on previous pharmacokinetic studies and on previous results of our

133 study group [16, 19, 21, 25-28]. On day six after infection, all mice were sacrificed; the  
134 kidneys were removed (both), weighed and homogenized aseptically. Homogenates were  
135 diluted tenfold; aliquots of 0.1 ml of the undiluted and diluted (1:10) homogenates were  
136 plated onto SDA plates and incubated at 35 °C for 48 h. The lower limit of detection was  
137 50 CFU/g of tissue. Statistical analysis of the kidney burden was performed using the  
138 Kruskal-Wallis test with Dunn's post-test for multiple comparisons [16, 19, 21].

139

### 140 **3. Results**

#### 141 **3.1. *In vitro* studies**

##### 142 **3.1.1. MIC results in RPMI-1640**

143 MIC values are presented in Table 1. Clinical isolates as well as the ATCC type strains  
144 were susceptible to caspofungin according to the revised CLSI breakpoints in RPMI in the  
145 BMD tests [3, 23]. Confirming the results from the preliminary experiments, isolates  
146 10781 and 34350 showed PG in RPMI. The DPL20 and DPL 18 strains were resistant to  
147 caspofungin [3, 23].

148 Etest MICs were 2- $\geq$ 8 times higher than MICs observed in the BMD (Table 1). PG was not  
149 observed, the inhibition zone was clear for all clinical isolates. *C. albicans* and *C.*  
150 *parapsilosis* ATCC strains were susceptible to caspofungin, while *C. krusei* ATCC strain  
151 was intermediate susceptible to caspofungin (Table 1 and Fig. 1) [3, 23]. MIC values for  
152 the two echinocandin resistant isolates were 2 and 32 mg/L (Table 1 and Fig. 1)

153

154

155 *3.1.2. MIC results in RPMI-1640+50% serum*

156 MIC values in the BMD and Etest methods were 2-16-fold higher in 50% serum when  
157 compared to the MICs obtained in RPMI without serum. MIC ranges of clinical isolates in  
158 50% serum in the BMD and Etest test were 0.125-0.25 and 0.25-0.5 mg/L, respectively.  
159 PG was never observed regardless of the method used (Table 1 and Fig. 1).

160

161 *3.1.3. Time kill results in RPMI-1640 and RPMI-1640+50% serum*

162 Maximum log decrease values are shown in Table 2; representative time-kill plots are  
163 shown in Fig. 2. The mean time to achieve 99.9% (T<sub>99.9</sub>) growth reduction from the  
164 starting inoculums at different caspofungin concentrations are shown in Table 3.

165 CFU decreases were isolate, concentration and medium dependent. Some isolates showed  
166 re-growth in both media (Table 2).

167 All isolates grew similarly well in both media; the mean time ranges of controls to growth  
168 1 log in RPMI and 50% serum were 8.66-9.77 and 8.9-10.89 hours, respectively.

169 In RPMI, caspofungin against 14171 and 35035 isolates produced fungistatic effect,  
170 however, at lower (0.25 and 1 mg/L) concentrations CFU decrease were higher than at 16  
171 and 32 mg/L concentrations (Table 2.). Against isolate 18799, caspofungin was fungicidal  
172 at  $\geq 4$  mg/L (Tables 2 and 3). In cases of 5265, 10781 and 34350 isolates typical PG was  
173 observed; fungicidal activity of caspofungin at lower concentrations and fungistatic effect  
174 at 16 and 32 mg/L. The T<sub>99.9</sub> values for these three isolates at 1-8 mg/L were short (<12 h,  
175 Table 3).

176 Caspofungin in 50% serum produced fungistatic effect against isolates 14171, 18799 and  
177 35035 (Fig. 2 and Table 2). Caspofungin killing activity in 50% serum was increased in



178 case of all three isolates which showed PG in RPMI-1640; all of these were killed at 4-32  
179 mg/L within 10 hours (Tables 2 and 3).

180

#### 181 *3.1.4. Killing rates in RPMI-1640*

182 Caspofungin killing rates ( $k$ ) were isolate and concentration dependent (Fig. 3). There was  
183 a trend producing higher  $k$  values at lower (0.25 or 1 mg/L) concentrations, while  $k$  values  
184 were very low at 32 mg/L. For all isolates  $k$  values were significantly higher at 1 than at 16  
185 and 32 mg/L ( $P < 0.001$ ). This paradoxical effect was the most prominent in cases of 35035  
186 and 5265 isolates where  $k$  values at 1 and 32 mg/L were 0.314 and 0.295 1/h, and 0.021  
187 and 0.011 1/h, respectively. Numerically, the highest  $k$  value was observed in case of  
188 10781 isolate, at 1 mg/L (0.961 1/h) (Fig. 3).

189

#### 190 *3.1.5. Killing rates in RPMI-1640 plus 50% serum*

191 Killing rate values at 0.25 mg/L were negative in cases of isolates 14171, 35035 and  
192 10781, indicating that growth occurred. For the remaining three isolates  $k$  value ranges at  
193 0.25 mg/L were 0.093-0.398 1/h (Fig. 3).

194 Killing rates for 18799, 35035, 5265, 10781 and 34350 isolates were concentration  
195 independent at 1-32 mg/L ( $p > 0.05$  for all comparisons). The lowest  $k$  value range was  
196 noticed in cases of isolate 18799 (0.085-0.109 1/h), while the highest range was found in  
197 case of isolate 10781 (0.882-0.985 1/h) (Fig. 3).

198 Isolate 14171 behaved differently. Killing rate values at 1, 4, 8 and 16 mg/L ( $k$  value range  
199 was 0.241-0.271 1/h) were significantly higher than at 32 mg/L ( $k$  value was 0.126 1/h)  
200 ( $P < 0.05-0.001$ ) (Fig. 3).

201 *3.1.6. Comparison of the killing kinetics at the same caspofungin concentrations in RPMI-*  
202 *1640 and 50% serum*

203 Killing rates at 0.25 mg/L were higher in RPMI than in 50% serum for all isolates ( $P < 0.05-$   
204  $0.001$ ). In cases of isolates 14171 and 18799  $k$  values were significantly higher at all tested  
205 concentrations in RPMI than in 50% serum, with the exception of 16 mg/L in case of  
206 14171 and 32 mg/L in case of 18799 isolate ( $P < 0.05-0.001$ ).

207 Caspofungin killing activity at 4-32 mg/L against the remaining isolates increased in 50%  
208 serum when compared to RPMI ( $P < 0.05-0.001$ ) with the exception of the concentration of  
209 8 mg/L in case of 5265, and of 4 and 32 mg/L in case of 34350 isolates.

210

211 **3.2. *In vivo* experiments**

212 All caspofungin doses decreased the fungal tissue burden for all tested clinical isolates  
213 (Fig. 4). One mg/kg caspofungin did not decrease significantly the fungal tissue burden in  
214 cases of isolates 18799, 10781 and 34350; moreover 2 mg/kg also proved to be ineffective  
215 in case of isolate 10781. However, caspofungin doses of 3, 5 and 15 mg/kg proved to be  
216 effective for all isolates ( $P < 0.05$  to  $0.001$ ). The largest caspofungin dose produced the  
217 highest mean fungal tissue burden decreases in cases of isolates 18799, 5265 and 10781.  
218 Numerically, caspofungin doses of 2, 3 and 2 mg/kg produced the lowest mean fungal  
219 tissue burden in cases of isolates 14171, 35035 and 34350, respectively (Fig. 4). However,  
220 statistically significant differences between the effective doses were never observed.

221 All doses but 1 mg/kg of caspofungin was effective against the ATCC type strain ( $P < 0.05$   
222 to  $0.001$ ); there was no significant differences between the effective doses. Against the  
223 echinocandin resistant strain caspofungin proved to be ineffective regardless of the doses  
224 (data not shown).

225

#### 226 **4. Discussion**

227 Frequency of invasive *Candida* infections is still increasing; though the non-albicans  
228 *Candida* species cause increasingly higher proportion of such infections, the most  
229 frequently isolated species is still *C. albicans* [3, 10-12]. Mean mortality rate is species  
230 dependent, the highest for *C. krusei* and *C. glabrata* (50-70%) and the lowest in case of *C.*  
231 *parapsilosis* (20-30%) [10-12]. The trend in the mortality did not change radically in the  
232 last decade, despite echinocandins were introduced into the antifungal armamentarium.  
233 Moreover, Lortholary and coworkers [13] noticed that the incidence and mortality of  
234 candidemia significantly increased in intensive care units (ICU), especially in case of *C.*  
235 *albicans* and *C. glabrata* infections. This trend is alarming because echinocandins usage  
236 increased from 4.6% to 48.5% between 2002 and 2010 among ICU patients in France [13].  
237 Fortunately, the rate of resistance to echinocandins is low worldwide [2-4, 13]. These data  
238 strongly suggest that optimization of antifungal therapy is crucial not only in cases of  
239 fluconazole or echinocandin resistant *Candida* isolates [2, 3, 10-12] but also in cases of  
240 drug-naïve (wild-type) isolates, which are probably highly susceptible to antifungals [2, 3,  
241 10-13].

242 Efficacy of echinocandins correlate with AUC/MIC (area under the concentration curve  
243 per MIC) or  $C_{max}/MIC$  (peak concentration per MIC), thus larger single or daily doses may  
244 lead to better clinical outcome [1, 2, 4, 5, 21, 28]. Betts and coworkers [29] used three  
245 times higher daily dose (150 mg) than the currently recommended (70 mg on day first  
246 followed by daily 50 mg) caspofungin doses for the treatment of adult patients with  
247 invasive candidiasis [2-4, 11, 29]. The daily 150 mg caspofungin produced numerically but  
248 not statistically higher cure rates among patients suffering from invasive *C. albicans* and  
249 *C. parapsilosis* infections when compared to the currently recommended dosage strategy,

250 without causing severe side effects [29]. As echinocandins are not the first choice in case  
251 of invasive *C. parapsilosis* infections our attention was drawn to the examination of the  
252 efficacy of higher caspofungin doses against *C. albicans* [1-4, 11, 16, 21, 29].

253 In our study we used six clinical isolates which showed diverse behavior in killing studies  
254 using RPMI as test medium; caspofungin was fungicidal against one isolate, was  
255 fungistatic against two isolates and three isolates showed PG. Decreased activity of  
256 caspofungin at higher (16-32 mg/L) concentrations was confirmed by the lower  $k$  values  
257 for all isolates in RPMI. Paradoxical growth is associated with the induction of cell wall  
258 salvage mechanisms by high caspofungin concentrations, which leads to increased amount  
259 of chitin in the cell wall compensating for the decreased  $\beta$ -glucan level [22, 30]. The  
260 detected PG of caspofungin against *C. albicans* is consistent with the results from other  
261 studies [6, 17, 22, 30].

262 In concordance with previous results [17, 22, 30], paradoxically decreased activity of  
263 higher concentration of caspofungin was eliminated by 50% serum both in MIC and in  
264 time-kill tests in cases of isolates 5265, 10781 and 34350. Fifty percent serum restored the  
265 killing activity of caspofungin at higher concentrations as revealed by  $k$  values. On the  
266 contrary, in case of isolate 14171 the killing rate was higher at 32 mg/L in RPMI than in  
267 50% serum.

268 The most notable finding of this study is that caspofungin showed concentration-  
269 independent activity in 50% serum at 1-32 mg/L against five out of six isolates as  
270 determined by the  $k$  values (the exception was again isolate 14171). However, the mean  $k$   
271 value ranges were isolate dependent. We expected that isolates showing higher  $k$  values of  
272 caspofungin either in RPMI or in 50% serum will produce better efficacy in a severely  
273 neutropenic murine model, especially at the largest dose [5, 28, 29]. Caspofungin produced  
274 concentration-dependent *in vivo* efficacy against the clinical isolates and the ATCC type

275 strain, i.e. concentrations 3, 5 and 15 mg/kg, but not 1 and 2 mg/kg were uniformly  
276 effective against all tested strains. Moreover, the largest (15 mg/kg) caspofungin dose not  
277 only did not produce significantly better fungal tissue burden decrease than the lower  
278 effective doses 3 and 5 mg/kg, but sometimes the highest numerical decrease was found in  
279 case of lower doses. These *in vivo* results also confirm that PG has no effect on the *in vivo*  
280 efficacy of caspofungin against *C. albicans* [2, 3, 17, 30]. The comparable efficacy of the  
281 safely effective doses is in line with the killing rate results in 50% serum. We have found  
282 similar correlation of *in vitro* and *in vivo* efficacy against *C. krusei* and *C. inconspicua*  
283 [19]. In agreement with previous findings, caspofungin was ineffective against the  
284 homozygous *fks1* mutant strain DPL20 [21, 31]. Thus, our *in vivo* experiments support the  
285 currently recommended caspofungin dosing strategy (70 mg on day first followed by daily  
286 50 mg), and indicate that dose-escalation has limited benefits against susceptible *C.*  
287 *albicans* in the clinic [2-4, 11, 29].

288 Currently, routine testing or reporting of CLSI and EUCAST MICs of caspofungin for  
289 different *Candida* species is not recommended, because of the unacceptably high  
290 interlaboratory variability in caspofungin MIC distribution in RPMI (wide MIC ranges  
291 coupled with bimodal MIC distributions) [2, 32]. Caspofungin MIC determination by Etest  
292 is not recommended either. Instead of caspofungin MIC determination both CLSI and  
293 EUCAST recommend testing for micafungin or anidulafungin MIC and these results  
294 should be interpreted for caspofungin susceptibility as well [2, 32].

295 In our study we used two types of serum-based susceptibility methods (BMD and Etest) to  
296 determine the MIC values for wild-type and echinocandin resistant strains. In order to  
297 detect PG by Etest, we used RPMI with and without 50% serum [24]; the serum-based  
298 Etest showed good correlation with serum-based BMD, hence, it may be applicable to  
299 determine caspofungin MICs both in case of susceptible and resistant isolates of *C.*

300 *albicans* (Fig. 1). Although we used low number of wide-type isolates for MIC testing, it is  
301 notable that the MIC range was narrower than in RPMI (Table 1). As serum-based  
302 susceptibility methods have not yet been standardized, they are not recommended currently  
303 for routine susceptibility testing of fungi [14, 23, 33]. The issues to be solved in the future  
304 include the optimal concentration of serum used in the tests, the origin (human or animal  
305 source), as well as the high cost of serum [33]. However, serum from animals (i.e. bovine  
306 serum) may replace human serum in the laboratories, decreasing the cost of serum-based  
307 MIC determinations [14]. Testing higher number of clinical *Candida* isolates with serum  
308 may provide a sufficient database to determine new epidemiological cutoff values and  
309 possible new clinical break-points for caspofungin against *Candida* species.

310 In spite of the extensive work on these antifungal agents, the mortality caused by invasive  
311 fungal infections is still increasing [10, 11, 12], highlighting the need for either new  
312 therapeutic agents, or other novel approaches. In parallel, the increasing resistance to  
313 antibacterial drugs induced research on enhancing the efficacy by modern delivery  
314 methods, e.g. linking the drugs with proteins [34] or incorporating them into nanoparticles  
315 or liposomes [35]. The latter approach also exists in antifungal therapy; three lipid  
316 formulations of amphotericin B are licensed. Research on lipid formulations of other  
317 antimycotics to improve efficacy [36] or to trap drugs at the site of infection [37] as well as  
318 on combination therapy [38] is also available.

319

320 **5. Conclusion**

321 Caspofungin activity was uniformly higher at lower than at higher concentrations in RPMI,  
322 with visible PG in MIC test or with prominent fungistatic activity at higher concentrations  
323 in time-kill tests. Fifty percent serum decreased the activity of caspofungin at 0.25 mg/L,  
324 but at 1-32 mg/L we noticed concentration independent killing activity. In a neutropenic  
325 murine model we demonstrated that caspofungin is equally effective against isolates  
326 showing and not showing PG *in vitro*, and dose escalation did not increase the efficacy of  
327 caspofungin against *C. albicans*. As serum-based susceptibility testing showed better  
328 predictive value of *in vivo* results than the currently recommended method, which is in  
329 accordance with the high protein binding of the echinocandin drugs, standardization of a  
330 serum-based protocol for susceptibility testing may be useful.

331

332 **6. Conflict of interests**

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334

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342



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483 Figure legends

484

485 Figure 1

486 Caspofungin MIC values in case of isolate 10781 as determined by Etest agar diffusion test. MIC values in  
487 RPMI-1640 (left) and RPMI-1640 supplemented with 50% serum (right) were 0.125 and 0.25 mg/L,  
488 respectively. Numbers on the Etest strip refer to caspofungin concentration in mg/L.

489

490 Figure 2

491 Time-kill curves of caspofungin against *Candida albicans* isolate 14171 in RPMI-1640 (A) and 50% serum (C),  
492 as well as 10781 isolate in RPMI-1640 (B) and 50% serum (D). The broken lines represent the fungicidal limit  
493 (3-log decrease).

494

495 Figure 3

496 Killing rates of caspofungin and the corresponding adjusted regression lines (dashed lines) against six *Candida*  
497 *albicans* clinical isolates in RPMI-1640 (RPMI) and RPMI-1640 plus 50% serum (Serum). Positive and negative  
498 *k* values indicate the decreases and increases, respectively, in viable cell numbers.

499

500 Figure 4

501 Kidney tissue burden of severely neutropenic BALB/c mice infected intravenously with six *C. albicans*  
502 isolates. Intraperitoneal caspofungin (CAS) (1, 2, 3, 5 and 15 mg/kg) daily treatment was started 24 hours  
503 after the infection. The bars represent the medians. Level of statistical significance compared to the control  
504 population is indicated at  $P < 0.05$  (\*),  $P < 0.01$  (\*\*) and  $P < 0.001$  (\*\*\*).

505

506 Figure 1

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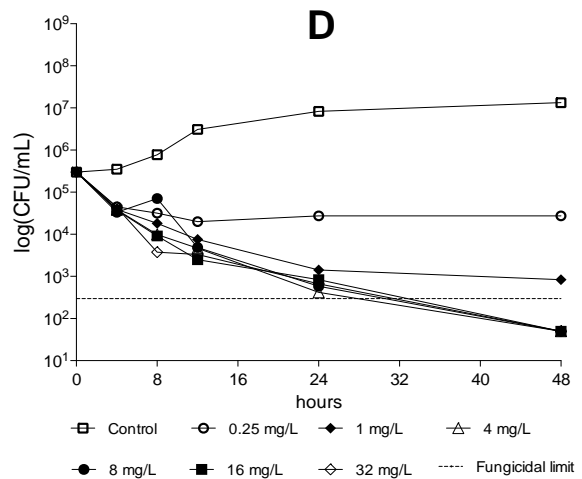
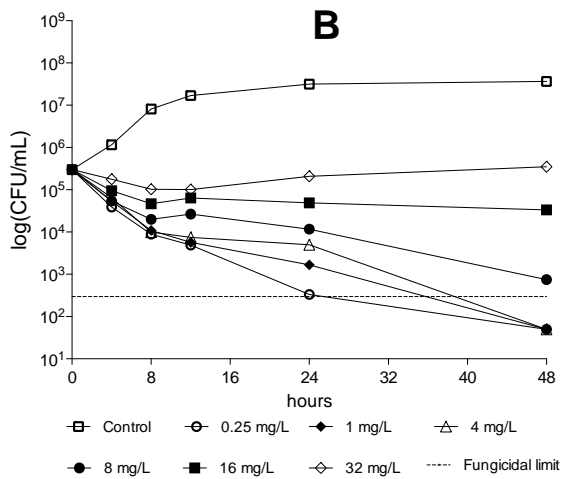
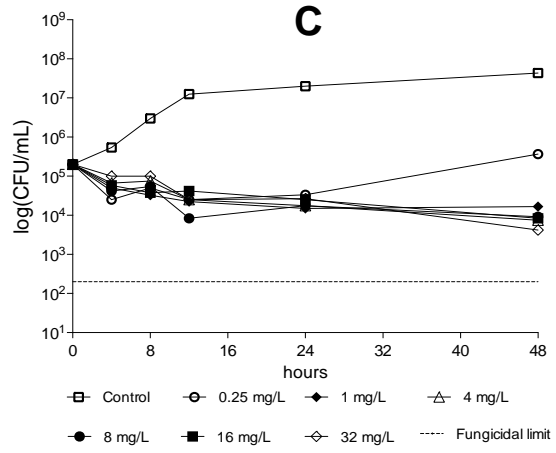
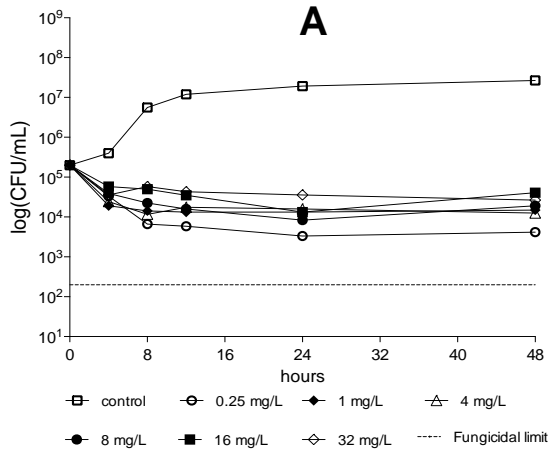
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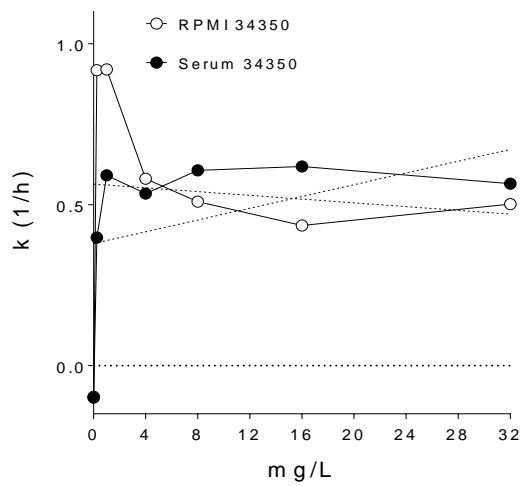
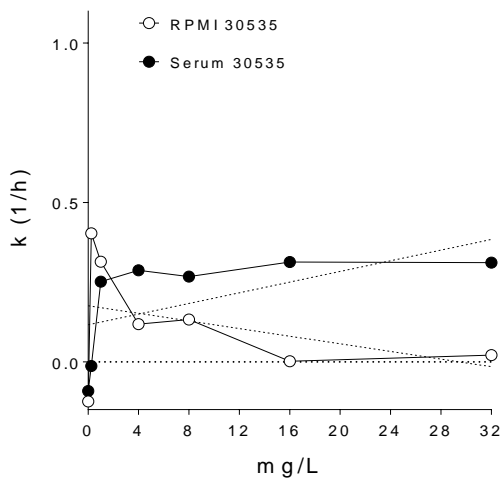
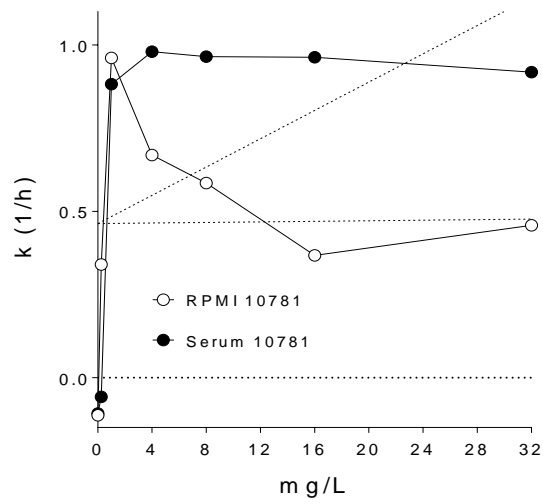
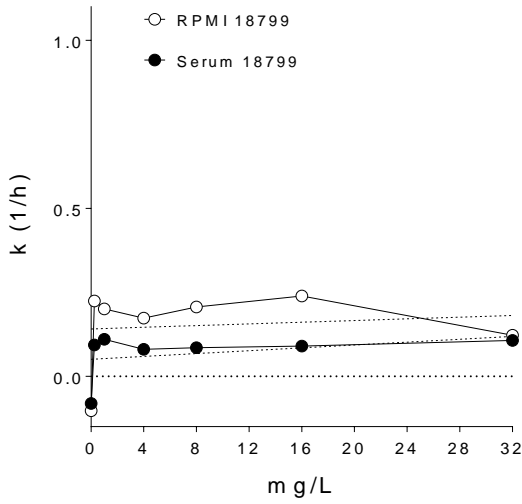
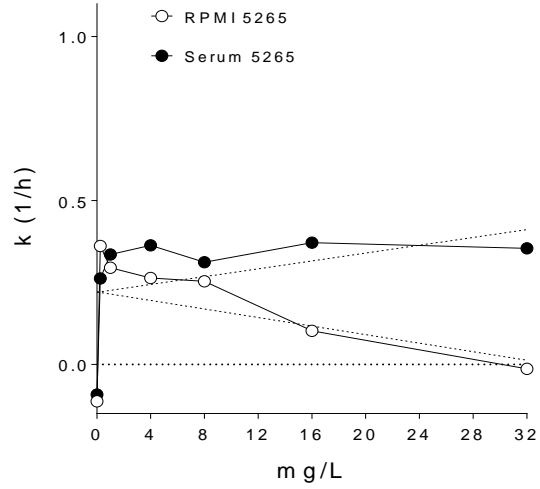
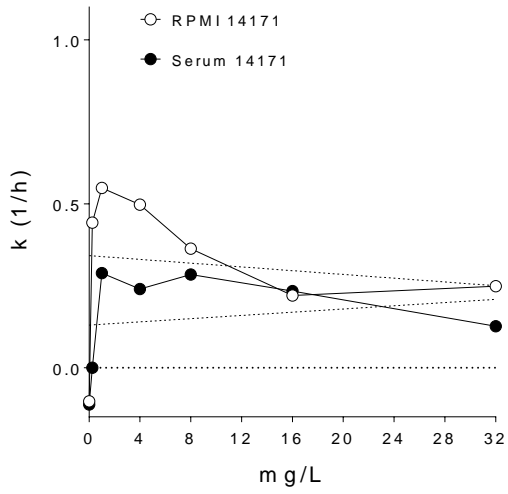
510 Figure 2

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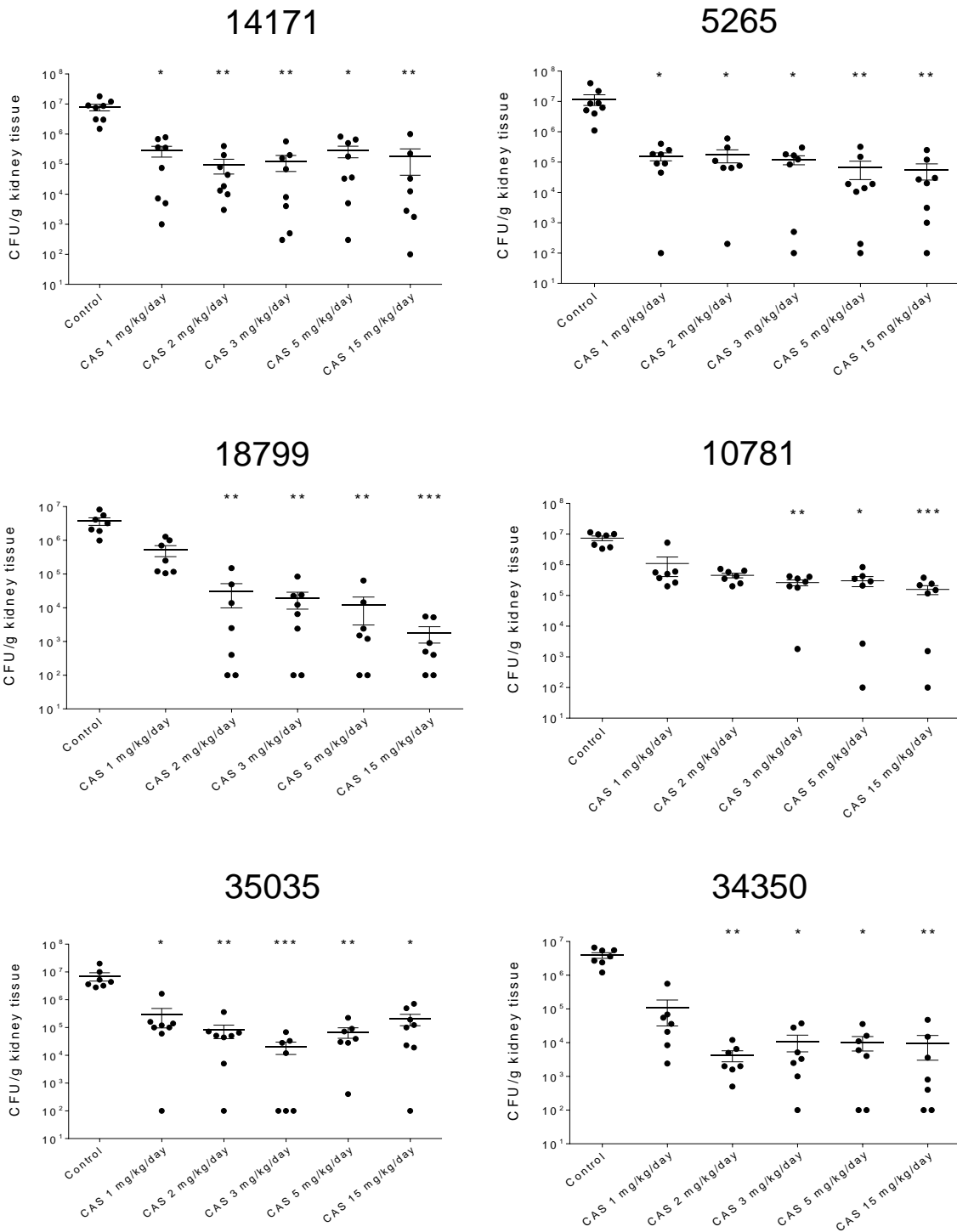
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523 **Table 1** *Candida albicans* isolates and MICs of caspofungin in RPMI-1640 (RPMI) and  
 524 RPMI-1640 plus 50 % serum (50% serum)

525

526

Isolates	Broth microdilution MICs (mg/L)		Ratio (MIC <sub>RPMI</sub> / MIC <sub>serum</sub> )	Etest MICs (mg/L)		Ratio (MIC <sub>RPMI</sub> / MIC <sub>serum</sub> )
	RPMI	50% serum		RPMI	50% serum	
<i>C. albicans</i> ATCC 10231	0.03	0.5	16x	0.125	0.5	4x
<i>C. albicans</i> 14171	0.03	0.12	4x	0.125	0.25	2x
<i>C. albicans</i> 18799	0.06	0.12	2x	0.125	0.5	4x
<i>C. albicans</i> 35035	0.03	0.25	8x	0.125	0.5	4x
<i>C. albicans</i> 5265	0.015	0.25	16x	0.03	0.5	16x
<i>C. albicans</i> 10781	0.03 <sup>PG</sup>	0.12	4x	0.12	0.25	2x
<i>C. albicans</i> 34350	0.015 <sup>PG</sup>	0.25	16x	0.12	0.25	2x
<i>C. albicans</i> DPL20	4	>32	>8x	>32	>32	1x
<i>C. albicans</i> DPL18	2	16	8x	2	32	16x
<i>C. krusei</i> ATCC 6258	0.25	2	8x	0.5	8	16x
<i>C. parapsilosis</i> ATCC 22019	0.5	1	2x	1	2	2x

527

528 PG: paradoxical growth

529

530 Table 2 Maximum log changes in log CFU/mL compared to starting inoculum in time-  
 531 kill studies in RPMI-1640 and RPMI-1640 plus 50 % serum (50% serum)

532

Isolate	Media	Maximum log decreases in CFU in time-killing experiments at the indicated caspofungin concentration (mg/L)					
		0.25	1	4	8	16	32
14171	RPMI-1640	-1.68	-1.12	-1.20	-1.38*	-0.69*	-0.88
	50% serum	-0.90*	-1.13	-1.43	-1.38*	-1.38	-1.68
18799	RPMI-1640	-2.60	-2.78	-3.00	-3.00	-3.00	-3.00
	50% serum	-0.89	-1.48	-1.71	-1.27	-1.88	-1.81
35035	RPMI-1640	-1.48	-2.68	-2.15	-1.15	-0.02	-0.38
	50% serum	-0.83*	-1.13	-2.10	-2.60	-1.38	-2.78
5265	RPMI-1640	-1.97*	-3.60	-3.60	-3.60	-1.68*	-2.08
	50% serum	-1.42*	-3.60	-3.60	-3.60	-3.60	-3.60
10781	RPMI-1640	-3.78	-3.78	-3.78	-2.60	-0.95	-0.16*
	50% serum	-1.18*	-2.65	-3.78	-3.78	-3.78	-3.78
34350	RPMI-1640	-3.52	-3.52	-3.52	-3.02	-2.09	-0.39*
	50% serum	-1.00	-3.52	-3.52	-3.52	-3.52	-3.52

533 \*Re-growth occurred

534

535

536 Table 3 Time (hours, h) to reach 99.9% (T99.9) growth reduction from the starting inocula  
 537 at different caspofungin concentrations (mg/L) in RPMI-1640 and RPMI-1640 plus 50 %  
 538 serum (50% serum). Subtraction signs (-) mean that fungicidal effect was not achieved.

539

Isolate number	Media	Time (h)					
		0.25	1	4	8	16	32
14171	RPMI-1640	-	-	-	-	-	-
	50% serum	-	-	-	-	-	-
18799	RPMI-1640	-	-	17.31	14.54	12.56	24.53
	50% serum	-	-	-	-	-	-
30535	RPMI-1640	-	-	-	-	-	-
	50% serum	-	-	-	-	-	-
5265	RPMI-1640	-	10.18	11.38	11.83	-	-
	50% serum	-	8.96	8.27	9.68	8.08	8.48
10781	RPMI-1640	8.82	3.12	4.48	-	-	-
	50% serum	-	-	3.06	3.11	3.11	3.27
34350	RPMI-1640	3.27	3.26	5.17	5.87	-	-
	50% serum	-	5.08	5.61	4.94	4.85	5.31

540

541

542