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Abstract

It was previously demonstrated that brief (≤1 h) exposures to echinocandins are as effective to kill *Candida albicans* cells as continuous 24-h exposure. However, killing rates after continuous and short (1 h) echinocandin exposures to *C. albicans* have not yet been evaluated in RPMI-1640 with and without 50 % serum. We evaluated four echinocandin susceptible *C. albicans* bloodstream isolates, ATCC 10231 type strain and an echinocandin-resistant isolate (DPL20, FKS F645P). Caspofungin MICs, time-kill and postantifungal effect (PAFE) tests were performed in RPMI-1640 with and without 50 % serum. Killing rates (*k* values) in time-kill and PAFE experiments were determined for each strain and concentration. In time-kill experiments, colony count decreases were isolate- and concentration-dependent at 0.25, 1, 4, 8, 16 and 32 mg/L in RPMI-1640, but concentration-independent at 1, 4, 8, 16 and 32 mg/L in 50 % serum. One-hour caspofungin exposure at 4, 16 and 32 mg/L resulted in CFU decreases comparable with the results obtained in time-kill experiments in RPMI-1640, but 50 % serum at 4, 16 and 32 mg/L allowed growth of all isolates (*k* values were negative) ($P < 0.05$–0.001). PAFE in 50 % serum decreased markedly at 4, 16 and 32 mg/L. Killing rates remained high and concentration-independent in 50 % serum in case of continuous but not in case of brief caspofungin exposure. As only a short growth inhibition without killing was observed in 50 % serum, clinical relevance of caspofungin PAFE in vivo is questionable.

Keywords (separated by '-'): Echinocandins - Serum-based susceptibility testing - Postantifungal effect

Footnote Information

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Killing Rates for Caspofungin Against *Candida albicans* After Brief and Continuous Caspofungin Exposure in the Presence and Absence of Serum

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Abstract It was previously demonstrated that brief (≤1 h) exposures to echinocandins are as effective to kill *Candida albicans* cells as continuous 24-h exposure. However, killing rates after continuous and short (1 h) echinocandin exposures to *C. albicans* have not yet been evaluated in RPMI-1640 with and without 50 % serum. We evaluated four echinocandin susceptible *C. albicans* bloodstream isolates, ATCC 10231 type strain and an echinocandin-resistant isolate (DPL20, FKS F645P). Caspofungin MICs, time-kill and postantifungal effect (PAFE) tests were performed in RPMI-1640 with and without 50 % serum. Killing rates (k values) in time-kill and PAFE experiments were determined for each strain and concentration. In time-kill experiments, colony count decreases were isolate- and concentration-dependent at 0.25, 1, 4, 8, 16 and 32 mg/L in RPMI-1640, but concentration-independent at 1, 4, 8, 16 and 32 mg/L in 50 % serum. One-hour caspofungin exposure at 4, 16 and 32 mg/L resulted in CFU decreases comparable with the results obtained in time-kill experiments in RPMI-1640, but 50 % serum at 4, 16 and 32 mg/L allowed growth of all isolates (k values were negative) ($P < 0.05$–0.001). PAFE in 50 % serum decreased markedly at 4, 16 and 32 mg/L. Killing rates remained high and concentration-independent in 50 % serum in case of continuous but not in case of brief caspofungin exposure. As only a short growth inhibition without killing was observed in 50 % serum, clinical relevance of caspofungin PAFE in vivo is questionable.

Keywords Echinocandins · Serum-based susceptibility testing · Postantifungal effect

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Introduction

Echinocandins (caspofungin, micafungin and anidulafungin) are reported to exhibit concentration-dependent fungicidal or fungistatic activity against the majority of *Candida* species. Additionally, echinocandins exert prolonged postantifungal effect (PAFE) after short (1 h) echinocandin exposure against many *Candida* species, including *C. parapsilosis* [1–4]. Recent in vitro findings with RPMI-1640 suggest that a very short (≤1 h) exposure to caspofungin kills *Candida* cells as effectively as a continuous 24-h exposure [5]. As echinocandins are highly protein-bound (≥96.5 %) agents (i.e., serum fundamentally

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influences killing activities of echinocandins) [6–8], we compared killing rates produced by short (1 h) and continuous (24 h) caspofungin exposure as well as PAFE in RPMI-1640 and 50 % serum against *C. albicans*.

Materials and Methods

Isolates

All *C. albicans* originated from blood samples (Table 1) and were identified as described previously [9]. ATCC 10231 and an echinocandin-resistant isolate (DPL20, FKS F645P) were also included in the study.

MIC Determination

Caspofungin (Sigma, Budapest, Hungary) MICs in RPMI-1640 with and without 50 % human serum (from a human male, type AB, Sigma, Budapest, Hungary) were determined using the CLSI broth macrodilution method [6–8, 10]. Caspofungin final concentration ranged between 0.015 and 32 mg/L. MICs were read after 24 h using the partial inhibition criterion [10].

Postantifungal Effect and Time-Kill Curves

PAFE was measured in both media simultaneously. As in our preliminary experiments 5-, 10- and 30-min exposures to 0.5, 1 or 2 mg/L caspofungin did not produce measurable PAFEs in 50 % serum (1–16 × MIC in 50 % serum), we used caspofungin at 4, 16 and 32 mg/L concentrations with a 60-min exposure time (brief caspofungin exposure) [3–5]. As the maximum administrable daily 150–200 mg caspofungin doses produce 30.4–40.6 mg/L geometric mean of peak concentrations in humans [11], the highest caspofungin concentration used in this study was 32 mg/L [6].

The starting inocula in PAFE experiments were 1–5 × 10^5 cells/ml [3–5]. After 1 h, the cells were collected by centrifugation at 1.500×*g* for 10 min and were washed three times with sterile saline, resuspended in 10 ml drug-free warm RPMI-1640 with and without 50 % human serum. Samples (100 µl) were removed at 0, 4, 8, 12 and 24 h, serially diluted tenfold, plated (4 × 30 µl) onto Sabouraud dextrose agar and incubated at 35 °C for 48 h [3–5].

For time-kill assays (continuous 24-h caspofungin exposure), test solutions were not centrifuged or washed. We used 0.25, 1, 4, 8, 16 and 32 mg/L caspofungin. In case of isolate DPL20, we used 4, 8, 16 and 32 mg/L caspofungin. Otherwise, the method was the same as described for PAFE [3–5]. All experiments were performed at least twice, and means of data are presented.

Fig. 1 Time-kill plots of caspofungin against four *Candida albicans* isolates (averages ± standard deviation) in RPMI-1640 (**a**) and 50 % serum (**d**) against ATCC 10231 type strain in RPMI-1640 (**b**) and 50 % serum (**e**), and against the echinocandin-resistant *C. albicans* DPL20 in RPMI-1640 (**c**) and 50 % serum (**f**). Clinical isolates and type strain were exposed to 0.25, 1, 4, 8, 16 and 32 mg/L, while DPL20 isolate was exposed to 4, 8, 16 and 32 mg/L of caspofungin for 24 h (continuous caspofungin exposure)

Table 1 *Candida albicans* isolates, MICs of caspofungin and the effect caspofungin in time-kill studies in RPMI-1640 (RPMI) and RPMI-1640 supplemented with 50 % serum (50 % serum)

Isolates	MIC (mg/L)		Effect in time-kill studies at concentrations shown (mg/L)	
	RPMI	50 % Serum	RPMI	50 % Serum
183	0.03	0.25	≥0.03 fungistatic	≥0.25 fungistatic
3666	0.03	0.125	≥0.03 fungistatic	≥0.25 fungistatic
12132	0.015	0.25	≥0.03 fungistatic	≥0.25 fungistatic
10920	0.03	0.125	≥0.03 fungistatic	≥0.25 fungistatic
ATCC 10231	0.03	0.5	≥0.03 fungistatic	≥0.5 fungistatic
DPL20	4	>32	≥16 fungistatic	No effect

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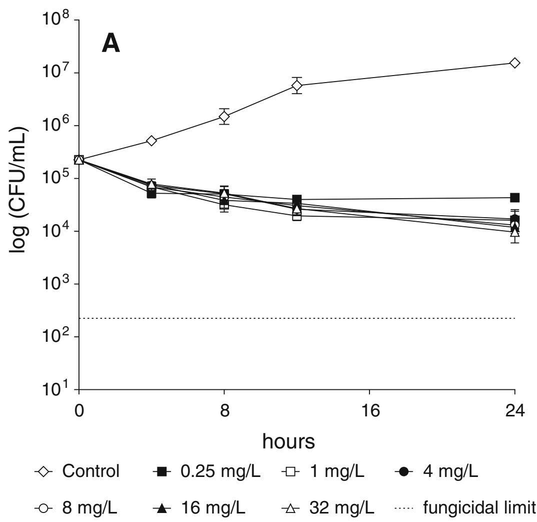

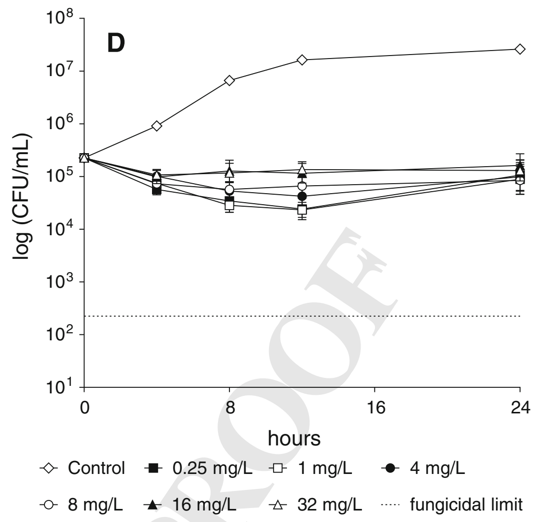

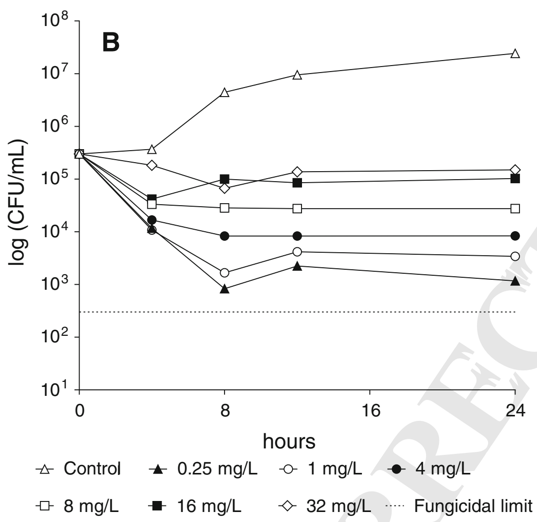

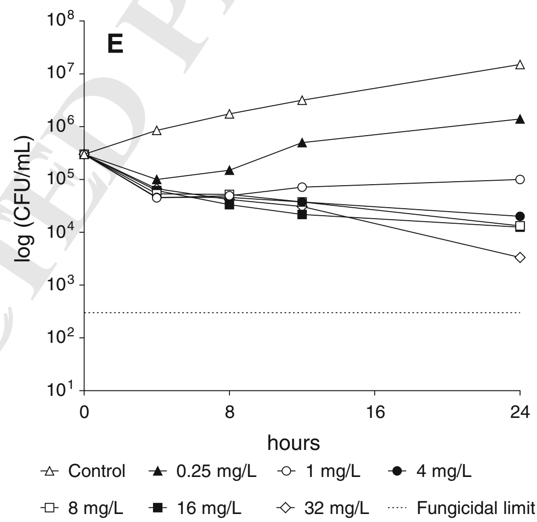

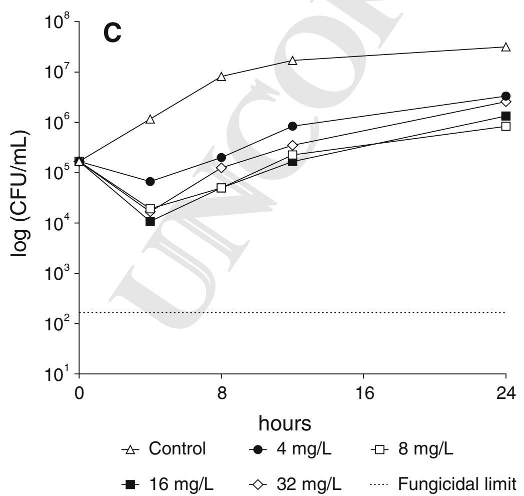

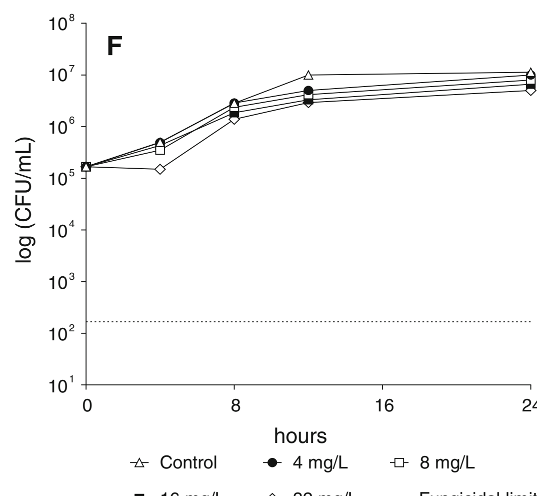

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107 Data Analysis

108 Killing kinetics was analyzed mathematically, as
109 described previously [6, 12]. An exponential equation
110 was fitted to the mean data at each time point:
111 $N_t = N_0 \times e^{-kt}$, where N_t is the number of viable
112 yeasts at time t, N_0 is the number of viable yeasts in the
113 initial inoculum, k is the killing rate and t is the
114 incubation time. Negative k values indicate growth,
115 and positive k values indicate killing. The goodness of
116 fit for each isolate was assessed by the r^2 value (>0.8)
117 [6, 12]. PAFE was defined as the difference between
118 the time required for control and test isolates to grow 1
119 $\log_{10}$ following drug removal [3–5].

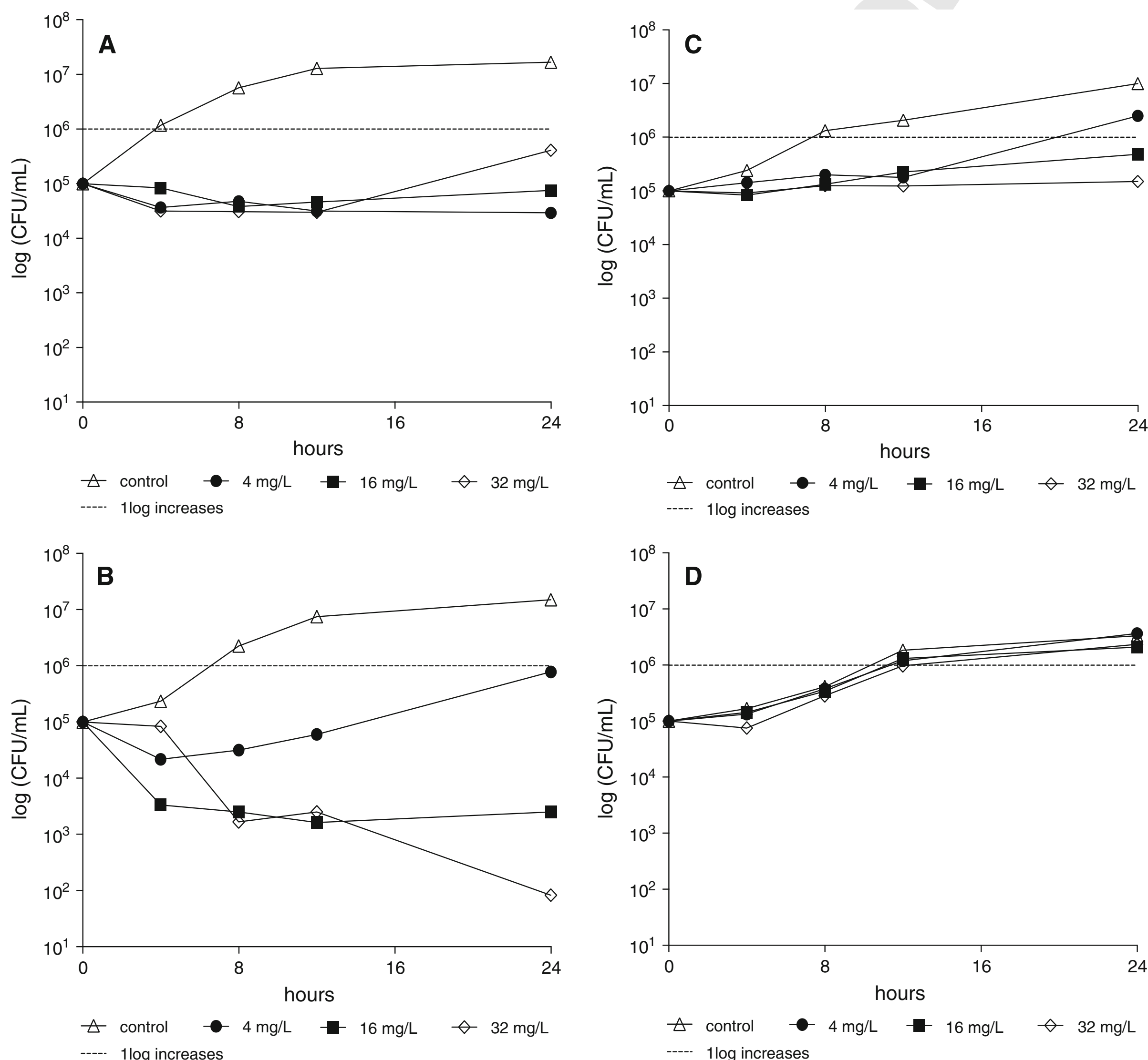

Fig. 2 Postantifungal effect curves of caspofungin against *Candida albicans* isolate 183 in RPMI-1640 (**a**) and 50 % serum (**c**), and against *C. albicans* isolate 3666 in RPMI-1640 (**b**) and 50 % serum (**d**). Isolates were exposed to 4, 16 and 32 mg/L of caspofungin for 1 h (brief caspofungin exposure)

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120 One-way ANOVA with Tukey's posttesting was
121 used to analyze differences in killing rates by
122 concentrations in either RPMI-1640 or 50 % serum
123 [6]. Paired *T* test was used to compare the effect of the
124 medium as well as the killing and growth rates in time-
125 kill and PAFE experiments at the same drug
126 concentration.

Results 127

Susceptibility 128

MIC values are presented in Table 1. Paradoxical 129
growth was not observed. Clinical isolates and 10231 130
ATCC strain were susceptible to caspofungin 131

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Table 2 Postantifungal effect (PAFE) of caspofungin against *Candida albicans* isolates at 4, 16 and 32 mg/L in RPMI-1640 (RPMI) and RPMI-1640-50 % serum (50 % serum)

Isolate number	Medium	PAFE in hours		
		4 (mg/L)	16 (mg/L)	32 (mg/L)
183	RPMI-1640	>19.34	>19.34	14.81
	50 % Serum	2.27	10.14	>18.79
3666	RPMI-1640	7.40	>19.23	>19.23
	50 % Serum	0	0.79	1.01
10920	RPMI-1640	>19.05	>19.05	>19.05
	50 % Serum	0	0.24	0.24
12132	RPMI-1640	14.21	13.84	7.65
	50 % Serum	0.60	0.95	3.46
ATCC 10231	RPMI-1640	4.89	>19.88	>19.88
	50 % Serum	0.09	0.34	1.39
DPL20	RPMI-1640	0	0	0
	50 % Serum	0	0	0

Table 3 Maximum log changes in log CFU/mL compared to starting inoculum in time-kill and postantifungal (PAFE) studies in RPMI-1640 and RPMI-1640-50 % serum (50 % serum)

Isolate number	Media	Maximum log decreases in CFU in time-killing and PAFE experiments at the indicated caspofungin concentration					
		4 mg/L		16 mg/L		32 mg/L	
		Time kill	PAFE	Time kill	PAFE	Time kill	PAFE
183	RPMI-1640	−1.1	−0.53	−1.18	−0.42	−0.75	−0.48
	50 % serum	−1.06	+0.37	−0.9	−0.08	−0.9	−0.04
3666	RPMI-1640	−1.38	−0.66	−0.54	−1.6	−0.34	−2.08
	50 % serum	−1.40	+0.13	−1.12	+0.12	−2.68	−0.12
10920	RPMI-1640	−1.23	−1.23	−1.22	−1.48	−0.69	−1.48
	50 % serum	−0.69	+0.49	−1.46	+0.38	−1.43	+0.64
12132	RPMI-1640	−0.44	−0.30	−0.19	−0.27	−0.23	−0.62
	50 % serum	−1.56	+0.67	−1.71	+0.66	−1.86	+0.27
ATCC 10231	RPMI-1640	−1.56	−1.48	−0.55	−2.08	−1.65	−1.78
	50 % serum	−1.18	+0.26	−1.38	+0.09	−1.95	+0.08
DPL20	RPMI-1640	−0.39	+0.61	−1.19	+0.78	−1.00	+0.37
	50 % serum	+0.81	+0.84	+0.69	+0.57	−0.05	+0.71

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A
Clinical isolates RPMI
k (1/h)
mg/L

D
Clinical isolates serum
k (1/h)
mg/L

B
DPL20 RPMI
k (1/h)
mg/L

E
DPL20 serum
k (1/h)
mg/L

C
ATCC 10231 RPMI
k (1/h)
mg/L

F
ATCC 10231 serum
k (1/h)
mg/L

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◀**Fig. 3** Killing rates of caspofungin and the corresponding fitted regression lines (*dashed lines*) in time-kill experiments against four *C. albicans* isolates (averages ± standard deviation) in RPMI-1640 (**a**) and 50 % serum (**d**), against *C. albicans* DPL20 in RPMI-1640 (**b**) and 50 % serum (**e**), and against ATCC 10231 type strain in RPMI-1640 (**c**) and 50 % serum (**f**). Positive and negative *k* values indicate the decrease and increase, respectively, in viable cell numbers

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132 according to the revised CLSI break points in RPMI-
133 1640 [13]. As expected, the DPL20 isolate with a
134 prominent *fks* mutation was resistant to caspofungin
135 [13]. MIC values were 4- to 16-fold higher in the
136 presence of 50 % serum.

137 Time-Kill Experiments

138 In time-kill experiments, caspofungin was fungistatic
139 at ≥1–2× MIC in both media against the clinical
140 isolates as well as against the 10231 ATCC strain
141 (<99.9 % reduction in viable cell count compared to
142 the starting inoculum) (Table 1; Fig. 1a, b, d, e).
143 Against the resistant strain DPL20, caspofungin
144 produced a weak fungistatic effect in RPMI-1640
145 (Fig. 1C). In 50 % serum, the killing curves were
146 generally similar to control; at 32 mg/L, a negligible
147 reduction was observed after 4 h, but later the killing
148 curves again became similar to control (Fig. 1f).

149 Postantifungal Effect

150 The time required for control (drug-free) isolates to
151 grow 1 $\log_{10}$ was similar in both media (4.12–4.95 h in
152 RPMI-1640 and 4.69–5.17 h in 50 % serum). PAFE
153 plots for isolates 183 and 3666 in RPMI-1640 and in
154 50 % serum are shown in Fig. 2. In RPMI-1640,
155 clinical isolates and the ATCC 10231 strain showed
156 the inhibition of re-growth at 4, 16 and 32 mg/L for
157 4.89 to >19.34, 13.84 to >19.88 and 7.65 to >19.88 h,
158 respectively (Table 2). PAFE in 50 % serum
159 decreased markedly at 4, 16 and 32 mg/L concentra-
160 tions (Table 2; Fig. 2). Most isolates showed growth
161 in 50 % serum; colony count decreases occurred only
162 in cases of isolates 183 at 16 and 32 mg/L (Fig. 2c)
163 and 3666 at 32 mg/L (Fig. 2d) and only after 4 h and
164 were negligible (Table 3). In case of DPL20, isolate
165 PAFE was never observed regardless of media
166 (Table 2).

167 Comparison of Colony Count Changes in Time-
168 Kill and Postantifungal Effect Experiments

169 Maximum colony count changes compared to the
170 starting inocula in time-kill and PAFE experiments at
171 4, 16 and 32 mg/L are presented in Table 3.
172 Comparing the different media at the same con-
173 centrations in killing experiments, the CFU decrease
174 was generally higher in 50 % serum than in RPMI-
175 1640 (Table 3). Contrastingly, the CFU decrease in
176 PAFE experiments was significantly higher in RPMI-
177 1640 than in 50 % serum with all isolates and
178 concentrations ($P < 0.05$–0.001) (Table 3).
179 Comparing the colony counts reductions at the
180 same concentrations in PAFE and time-kill experi-
181 ments, we noticed comparable or sometimes higher
182 reductions (in cases of 10920 and 3666 isolates) in
183 PAFEs than that seen with continuous 24-h exposure
184 in RPMI-1640 (Table 3). However, 50 % serum
185 significantly decreased the PAFE killing for all tested
186 isolates ($P < 0.01$–0.001) (Table 3; Fig. 4a–e).

187 Killing Rates in Time-Kill Experiments

188 In time-kill experiments, killing activity of caspofun-
189 gin was significantly weaker at 16–32 mg/L than at
190 0.25, 1, 4 and 8 mg/L ($P < 0.05$–0.001) in RPMI-
191 1640 (mini-paradoxical effect) (Fig. 3a). However,
192 killing rates at 1–32 mg/L were concentration-inde-
193 pendent in 50 % serum against the susceptible isolates
194 (Fig. 3d). Similar effect was noticed in case of the
195 strain ATCC 10231 (Fig. 3c, f). In case of isolate,
196 DPL20 *k* values were negative (indicating the growth
197 instead of killing) regardless of media (Fig. 3b, e).

198 Killing Rates in Postantifungal Effect Experiments

199 In PAFE experiments, killing rates for clinical isolates
200 and the ATCC strain in RPMI-1640 were isolate- and
201 concentration-dependent (*k* values from −0.111 to
202 +1.019 1/h), while in 50 % serum, the *k* values
203 showed markedly narrower range (from −0.017 to −
204 0.185 1/h) (Fig. 4a–e).

205 **Discussion**

206 This study is the first in which killing rates in short and
207 continuous caspofungin exposures to *C. albicans* were

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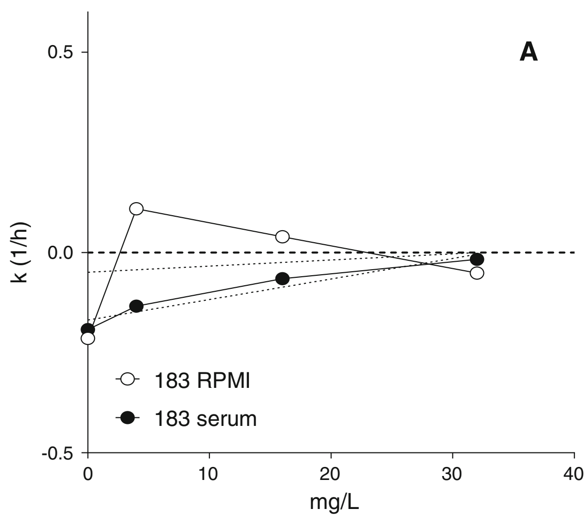

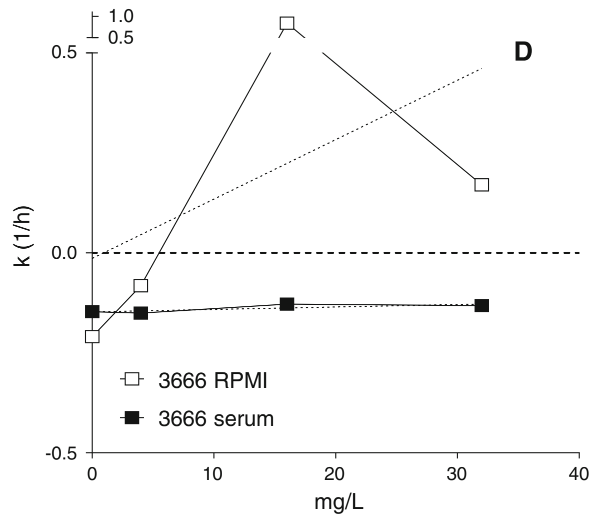

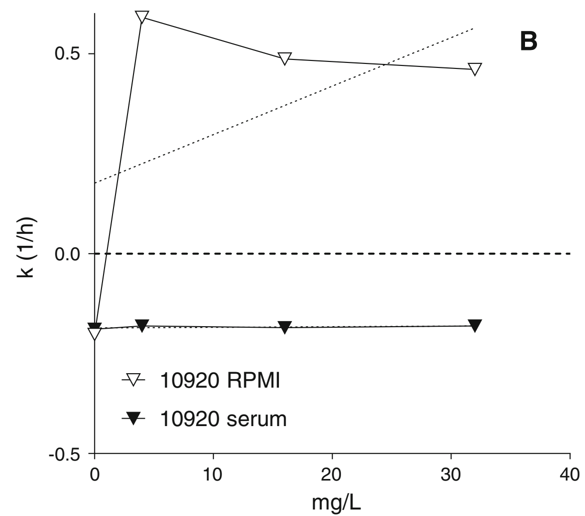

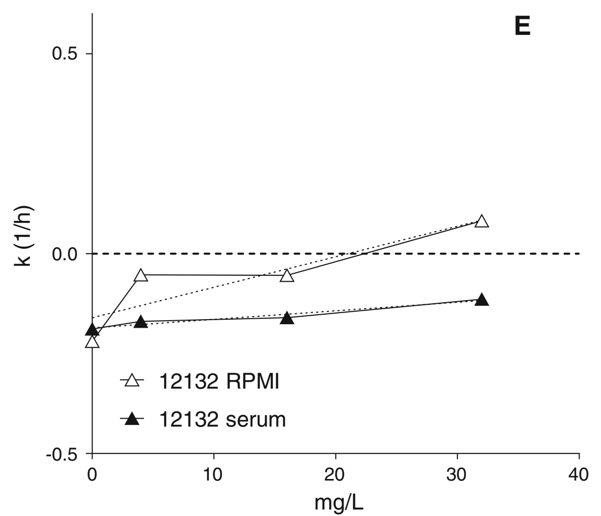

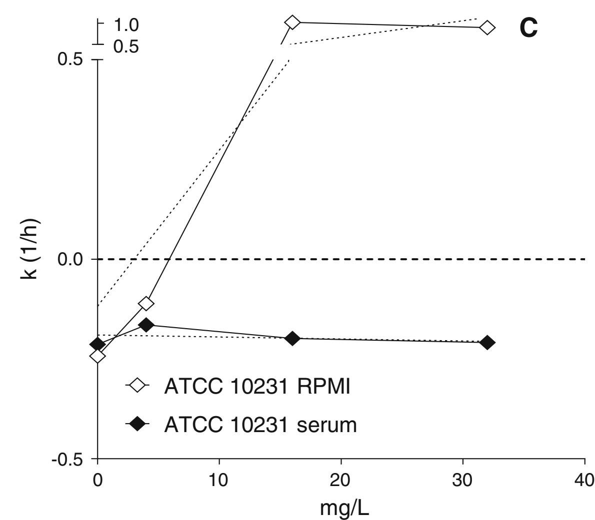

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◀**Fig. 4** Killing rates of caspofungin and the corresponding fitted regression lines (*dashed lines*) in PAFE experiments against 183 (**a**), 10920 (**b**), 3666 (**d**) and 12132 (**e**) *C. albicans* isolates in RPMI-1640 and 50 % serum, and against *C. albicans* ATCC 10231 type strain (**c**) in RPMI-1640 and 50 % serum. Positive and negative *k* values indicate the decrease and increase, respectively, in viable cell numbers. For panels C and D, the scale of the y axis was broken for better visualization of regression lines

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compared head to head in RPMI-1640 with and without 50 % serum. In agreement with previous results [3–5], the CFU decreases in time-kill and PAFE experiments were similar in RPMI-1640; however, at 16 and 32 mg/L, killing rates decreased. Decreased killing rates in time-kill experiments at 16 and 32 mg/L can be explained by the adaptive and compensatory response to high caspofungin concentrations in the fungal cells that limit killing effect and allow for the growth [14]. Addition of 50 % serum significantly decreased killing rates at 4, 16 and 32 mg/L in the PAFE experiments as compared to RPMI-1640, while the killing rates in time-kill experiments (continuous exposure) at ≥1 mg/L concentrations remained high and concentration-independent (i.e., killing rate reached its maximum at 1 mg/L). These findings are in accordance with our previous results, where killing rates of *C. krusei* and *C. inconspicua* did not differ significantly in 50 % serum at effective concentrations [6]. Moreover, 1-h exposure of *C. albicans* to 0.5, 1 and 2 mg/L of caspofungin in 50 % serum is not long enough to produce any growth inhibition, as opposed to what found with RPMI-1640 alone.

Louie et al. [15] demonstrated that tissues serve as drug reservoirs from which the drug is released slowly, explaining that serum caspofungin half-life tripled when both serum and tissues half-life were taken into account in the terminal half-life calculation. They concluded that the primary tissue reservoir rather than PAFE was responsible for the excellent in vivo activity of caspofungin [15]. Their results are in line with the present study, as 1-h exposure to caspofungin produced negative *k* values (growth) and significantly decreased PAFEs in 50 % serum.

PAFE is frequently regarded as a contributor to clinical efficacy of echinocandins [1–4, 13]. It is defined as prolonged growth inhibition following limited (generally 1 h) in vitro drug exposure; however, it must be noted that this is not equal to killing, as slower growth may also be regarded as prominent PAFE. While killing may directly lead to eradication, growth, even slower growth, of fungi still carries a risk of persistent infection and fungal re-growth. These facts should be taken into consideration when translating PAFE as a contributor to clinical efficacy. Present results strongly suggest that PAFE is lost in the presence of 50 % serum, even though marked PAFE is detected in RPMI-1640 after 5-min exposure [5]. The negligible PAFEs found in 50 % serum indicate that prolonged in vitro PAFEs (in RPMI-1640), frequently interpreted as a contributor to better clinical efficacy [1–4], may be less important in vivo (better mimicked by the serum-containing medium) at least against *C. albicans*. Whether these also apply for non-albicans species is to be answered by further studies.

In summary, continuous but not brief caspofungin exposure produced measurable killing rates against *C. albicans* clinical isolates in killing studies in the presence of 50 % serum. PAFE after brief exposure to caspofungin (and probably to other echinocandins), even when marked, may play a limited role in the excellent clinical efficacy of echinocandins.

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