Five-Minute Exposure to Caspofungin Results in Prolonged Postantifungal Effects and Eliminates the Paradoxical Growth of *Candida albicans*[▽]

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We studied the impact of brief caspofungin exposures on postantifungal effects (PAFEs) and paradoxical effects for five *Candida albicans* isolates. In time-kill studies, caspofungin at 4× and 16× the MIC resulted in significant killing of all isolates. Caspofungin at 8 μg/ml resulted in lower levels of killing, and paradoxical effects were evident with 4 isolates. Caspofungin exposures of 5 to 60 min caused prolonged, concentration-dependent killing that approached or exceeded the results seen with time-kill experiments and eliminated paradoxical growth.

Caspofungin, an inhibitor of 1,3-β-D-glucan synthesis, demonstrates concentration-dependent fungicidal activity *in vitro* (5, 8) and *in vivo* (1, 12) against *Candida* spp. Our laboratory and others have demonstrated that caspofungin exerts prolonged postantifungal effects (PAFEs) on *Candida* spp. following *in vitro* exposures of 1 h (5, 7, 9, 14). At the same time, caspofungin exerts paradoxical effects *in vitro*, in which the growth of certain *Candida* isolates is increased at caspofungin concentrations above the MIC (19). To date, the relationships between PAFEs and paradoxical effects have not been investigated. The objective of the present study was to evaluate the impact of brief exposures to caspofungin (ranging from 5 to 60 min) on both PAFEs and paradoxical growth of *Candida albicans*.

Four randomly chosen *C. albicans* bloodstream isolates collected from the Clinical Microbiology Laboratory at the University of Pittsburgh Medical Center and *C. albicans* SC5314 were included in this study (10). Caspofungin time-kill and PAFE experiments were performed in parallel (5, 16, 17). In total, four test solutions were used, and consisted of RPMI 1640 alone (control) and RPMI 1640 plus caspofungin at concentrations of 4× the MIC, 16× the MIC, and 8 μg/ml. We selected 8 μg/ml to simulate peak serum concentrations *in vivo* (15). After incubation at 35°C with agitation for 5, 15, 30, and 60 min, the cells were washed with saline three times and then resuspended in 20 ml of medium (RPMI 1640) and reincubated at 35°C (PAFE experiments). For time-kill assays, test solutions were not centrifuged or washed. All tubes were sampled at 0, 2, 4, 8, 12, and 24 h by removing 500 μl of the reaction mixture and enumerating CFU on Sabouraud dextrose agar (SDA) plates. All experiments were performed at least twice.

Paradoxical effects were defined as ≥ 1 log₁₀ CFU/ml of

greater growth in the presence of 8 μg/ml of caspofungin compared to 4× and 16× the MIC (19). The killing effect of caspofungin was calculated as the difference between the starting inoculum and the concentrations of isolates at specific time points. In time-kill experiments, caspofungin at concentrations of 4× and 16× the MIC caused reductions in the concentration of the starting inocula of all tested isolates after 24 h of incubation (Table 1 and Fig. 1). In contrast, caspofungin at 8 μg/ml (i.e., 64× to 256× the MIC values) did not reduce the concentration of the starting inocula of isolates 1 and 3 (Table 1). For four of five isolates, paradoxical effects were evident, as caspofungin concentrations at 4× and 16× the MIC resulted in a greater killing effect than 8 μg/ml. Moreover, for two isolates, caspofungin at 4× the MIC caused greater killing than 16× the MIC. Overall, the killing effects of caspofungin at 4× the MIC were significantly greater than those seen at 8 μg/ml (mean log₁₀ reduction in the starting inocula, 2.25 log versus 0.50 log [$P = 0.048$; Wilcoxon signed-rank test]).

For each isolate, brief (5, 15, 30, and 60 min) exposures to caspofungin during PAFE experiments resulted in concentration-dependent killing (Table 1 and Fig. 2) that persisted for 24 h. At 4× the MIC, killing of four isolates after brief exposures was less than that seen during continuous drug exposure in time-kill experiments. At 16× the MIC and 8 μg/ml, however, PAFEs on three and four isolates, respectively, resulted in greater killing than continuous drug exposure. In general, maximal PAFEs on each of the isolates were achieved after exposures as short as 5 or 15 min (Table 1). Moreover, removal of caspofungin (8 μg/ml) after 5 to 60 min abolished paradoxical effects on all four isolates, as observed in time-kill experiments. Indeed, 5-min exposures to caspofungin at 8 μg/ml resulted in significantly greater mean killing effects than continuous exposure to caspofungin at 8 μg/ml (mean log₁₀ reduction in the starting inocula, 2.48 log versus 0.30 log [$P = 0.01$; Wilcoxon signed-rank test]).

To conclusively demonstrate that the killing effects we observed after brief caspofungin exposures stemmed from PAFEs rather than representing an artifact of the experimental methods, we conducted three experiments in parallel: (i) stan-

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TABLE 1. Caspofungin MICs and reductions in *Candida albicans* starting inocula during 24-h time-kill and postantifungal effect (PAFE) experiments

Isolate	Caspofungin MIC ($\mu\text{g/ml}$)	Caspofungin concn ($\mu\text{g/ml}$)	Log kill (CFU/ml) for indicated experiment ^a				
			Time-kill	PAFE (5 min)	PAFE (15 min)	PAFE (30 min)	PAFE (60 min)
1	0.06	0.25 (4 $\times$ MIC)	2.14	1.38	0.96	0.63	No kill
		1 (16 $\times$ MIC)	0.65	2.12	2.09	2.06	1.34
		8	No kill, PG	2.01	2.51	2.44	2.39
2	0.06	0.25 (4 $\times$ MIC)	1.21	No kill	0.09	0.07	No kill
		1 (16 $\times$ MIC)	1.20	1.41	1.16	1.03	No kill
		8	2.15	2.31	2.67	1.76	1.24
3	0.125	0.5 (4 $\times$ MIC)	1.50	1.58	1.28	1.86	1.24
		2 (16 $\times$ MIC)	1.53	2.43	2.10	1.76	2.26
		8	No kill, PG	2.79	1.81	2.40	3.04
4	0.03	0.12 (4 $\times$ MIC)	1.57	No kill	0.99	0.55	No kill
		0.5 (16 $\times$ MIC)	1.44	No kill	1.29	0.51	0.02
		8	0.49, PG	1.66	0.83	0.76	1.66
SC5314	0.125	0.5 (4 $\times$ MIC)	4.81	2.45	1.82	0.88	2.38
		2 (16 $\times$ MIC)	1.26	2.10	2.87	2.13	2.14
		8	0.67, PG	3.61	2.04	2.46	2.71

^a No kill indicates that, after 24 h of incubation, there was no significant reduction from the starting inoculum value. PG, paradoxical growth, defined as greater growth ($\geq 1 \log_{10}$) in the presence of caspofungin administered at 8 $\mu\text{g/ml}$ compared to 4 $\times$ or 16 $\times$ the MIC during time-kill experiments. PAFE values indicate log kill (CFU/ml) as determined by the reduction in the starting inocula (at time zero) after 24 h of incubation.

dard time-kill experiments using continuous exposure to caspofungin; (ii) standard PAFE experiments in which caspofungin was washed out after brief exposures; and (iii) caspofungin reexposure experiments in which the drug was reapplied to cells at the original concentration after completion of the washout steps of PAFE experiments. Over 24 h, the kill curves for time-kill and caspofungin reexposure experiments were similar for caspofungin at 16 $\times$ the MIC and 8 $\mu\text{g/ml}$, whereas maximal killing was observed after brief exposures (Fig. 3). Paradoxical growth of the four relevant isolates was apparent during both time-kill and reexposure experiments but was eliminated during PAFE experiments.

In this study, we showed that caspofungin caused prolonged killing of *C. albicans* isolates in a concentration-

dependent manner. Indeed, exposures to caspofungin for as little as 5 or 15 min resulted in killing over 24 h that approached or, in some instances, exceeded that seen with continuous drug exposure during time-kill experiments. These findings extended previous observations from our laboratory and others showing that 1 h of exposure to caspofungin resulted in significant PAFEs (5, 8). At the same time, we showed that brief exposures to caspofungin at 8 $\mu\text{g/ml}$, a typical peak concentration within human serum, eliminated the paradoxical growth of all isolates that manifested the phenomenon during time-kill experiments.

The potent PAFEs stemming from brief caspofungin exposures suggest that the drug rapidly accesses its target and causes sustained inhibition of the β -1,3-D-glucan synthase enzyme complex. Studies of bacterial protein and nucleic acid synthesis inhibitors have demonstrated that the duration of postantibiotic effects is determined by the period of time needed for the drugs to dissociate from their targets (20, 21). As such, the PAFEs in this study were most likely due to ongoing direct interactions between caspofungin and β -1,3-D-glucan synthase. Alternatively, the drug may be able to intermittently associate with its target from sequestered sites within the plasma membrane or elsewhere in the cell. Regardless of the particular mechanisms by which PAFEs are mediated, our data advocate a model in which the maximal activity of caspofungin against a given *C. albicans* isolate is attained at some threshold determined by a combination of the duration of drug exposure, drug concentration, and target affinity. In the face of caspofungin exposure above this threshold, such as that resulting from constant exposure to high drug concentrations, adaptive and compensatory responses may be activated that limit further killing and promote growth. By the same token, a brief exposure to a sufficient concentration of caspofungin may re-

FIG. 1. Time-kill curve of a representative *C. albicans* isolate. Paradoxical effects were evident, as growth was greater after 24 h in the presence of caspofungin at 8 $\mu\text{g/ml}$ than at 4 $\times$ or 16 $\times$ the MIC. The *C. albicans* strain is listed as isolate 1 in Table 1 (MIC = 0.06 $\mu\text{g/ml}$). As detailed in Table 1, similar results were observed for other isolates.

FIG. 2. Postantifungal effects of caspofungin on a representative *C. albicans* isolate. Drug exposures of 5, 15, 30, and 60 min (Fig. 2a, b, c, and d, respectively) during PAFE experiments resulted in significant reductions in the concentrations of the starting inocula that persisted for 24 h. Paradoxical effects were eliminated, and overall killing was at least as extensive as that seen during the time-kill experiments (Fig. 2e). Results for this isolate (isolate 3 in Table 1) are representative of those observed for other isolates.

sult in killing equivalent to that seen with longer exposures to lesser concentrations.

At present, the standard dosage of caspofungin for the treatment of candidiasis is a 70-mg loading dose, followed by 50 mg administered daily (15). Caspofungin's concentration-dependent PAFEs that persist for 24 h suggest that less-frequent administration of higher drug doses may be at least as effective as conventional regimens (3). In fact, as predicted from pharmacodynamic (PD) studies (2, 17), once-weekly administration of another echinocandin, micafungin, was shown to be as efficacious as daily administration in a murine model of systemic candidiasis (11). In this regard, it is notable that PD targets for caspofungin are readily achievable *in vivo* despite less-frequent administration due to the tolerability of high doses (4) and the persistence of therapeutic concentrations in tissues (13). In addition to direct killing, caspofungin PAFEs may provide indirect benefits *in vivo*, such as rendering cells more susceptible to phagocytosis and cell wall stress and decreasing adherence to host epithelial cells (16, 17). Alternative dosing strategies for caspofungin and other echinocandins merit further

investigation, including assessments of the impact on toxicity, cost, and emergence of resistance.

The clinical relevance of caspofungin paradoxical effects remains uncertain. We recently demonstrated that paradoxical effects were eliminated in 50% human serum, suggesting that they are unlikely to influence the treatment of candidemia (19). Our demonstration that paradoxical growth was eliminated during PAFE experiments further argues against a major clinical impact unless extremely high caspofungin concentrations are maintained throughout the dosing interval. Indeed, in a mouse model of systemic candidiasis, only one of four *C. albicans* isolates that demonstrated a paradoxical effect *in vitro* showed a similar pattern *in vivo*, with a higher fungal burden recovered from mice treated with high-dose caspofungin (20 mg/kg of body weight) (6). Moreover, the apparent paradoxical growth was not reproducible in subsequent experiments (6). Furthermore, in two recent clinical trials, there were no differences in responses to echinocandins among patients with candidiasis who were subjected to high- or low-dose regimens (150 mg/day versus 100 mg/day of micafungin and 150 mg/day

FIG. 3. Effect of cell washings followed by reexposure of cells to caspofungin on growth of a representative *C. albicans* isolate. Isolates were tested in three parallel experiments. These included (a) a time-kill experiment with continuous exposure to caspofungin, (b) a PAFE experiment with brief exposures to caspofungin (PAFE), and (c) an experiment consisting of reexposure to caspofungin following PAFE and cell washing. Reexposure to caspofungin at 16 $\times$ the MIC and 8 μ g/ml resulted in growth curves similar to those seen with constant exposure during time-kill experiments, including paradoxical effects. The results shown are representative of the results observed for other isolates.

versus a 70-mg load followed by administration of caspofungin at 50 mg/day) (4, 18). On balance, therefore, the preponderance of evidence suggests that paradoxical effects are not likely to be clinically relevant.

In conclusion, the current study opens the door to future

investigations to explore the mechanisms by which caspofungin and other echinocandins rapidly exert their profound antifungal activity against *C. albicans*. In particular, studies are needed to improve understanding of the interaction between these agents and their target, the enzyme kinetics of β -1,3-D-glucan synthase, and the compensatory responses that are triggered by various degrees of echinocandin exposure. Results from these studies would have important implications for both the treatment of candidiasis and our understanding of *Candida* cell wall biology.

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