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Effects of Caspofungin against *Candida guilliermondii* and *Candida parapsilosis*

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The in vitro activity of caspofungin (CAS) was investigated against 28 yeast isolates belonging to *Candida albicans* ($n = 5$), *Candida guilliermondii* ($n = 10$), and *Candida parapsilosis* ($n = 13$). CAS MICs obtained by broth dilution and Etest methods clearly showed a rank order of susceptibility to the echinocandin compound with *C. albicans* > *C. parapsilosis* > *C. guilliermondii*. Similarly, time-kill assays performed on selected isolates showed that CAS was fungistatic against *C. albicans* and *C. parapsilosis*, while it did not exert any activity against *C. guilliermondii*. In a murine model of systemic candidiasis, CAS given at doses as low as 1 mg/kg of body weight/day was effective at reducing the kidney burden of mice infected with either *C. albicans* or *C. guilliermondii* isolates. Depending on the isolate tested, mice infected with *C. parapsilosis* responded to CAS given at 1 and/or 5 mg/kg/day. However, the overall CFU reduction for *C. guilliermondii* and *C. parapsilosis* was approximately 100-fold less than that for *C. albicans*. Our study shows that CAS was active in experimental systemic candidiasis due to *C. guilliermondii* and *C. parapsilosis*, but this activity required relatively high drug dosages.

The frequency of invasive mycoses due to opportunistic fungal pathogens has increased dramatically over the past 2 decades, and now *Candida* spp. rank as the fourth most common cause of nosocomial bloodstream infections (17, 21). Although *Candida albicans* is the organism most often associated with serious fungal infections, other *Candida* spp. have emerged as clinically important pathogens associated with opportunistic infections (17, 21). *Candida parapsilosis* is the second most common yeast species isolated from blood in Europe and South America (17, 20). It is particularly associated with bloodstream infections in neonates and with catheter-associated candidemia and intravenous hyperalimentation (17, 20).

Another emerging species of *Candida* is *Candida guilliermondii* (17). It has been shown to cause hematogenously disseminated candidiasis, and it is considered intrinsically less susceptible to amphotericin B (AMB) than other *Candida* spp. are (17).

Although both *C. parapsilosis* and *C. guilliermondii* show a reduced innate virulence compared with *C. albicans*, there is a common trait regarding their susceptibility patterns to caspofungin (CAS), an echinocandin antifungal agent that has potent activity against many fungal species, including *Candida* spp. (1–4, 8–12, 22, 23). Clinical studies have shown that CAS is at least as active as AMB and fluconazole in the treatment of invasive candidiasis (4, 12, 22, 23). However, in vitro susceptibility data on CAS indicate that *C. guilliermondii* and *C. parapsilosis* are the least susceptible species in the genus *Candida* (2, 7, 15, 18). In general, CAS MICs reported for these two species of *Candida* are from 8 to 32 times higher than those for *C. albicans* (7, 18). Whether this finding could be of clinical relevance is not still understood very well. Therefore, in this study, we analyzed the effects of CAS against these two species of *Candida* in either in vitro or in vivo experiments.

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MATERIALS AND METHODS

Isolates. A total of 28 strains of *Candida* spp. were used in this study. Control organisms included *Candida albicans* ATCC 90029, *C. albicans* SC5314, and *Candida parapsilosis* ATCC 22019. An additional 3 clinical isolates of *C. albicans*, 10 isolates of *Candida guilliermondii*, and 12 isolates of *C. parapsilosis* were investigated. All clinical isolates were recovered from blood, and each represented a unique isolate from a patient. Yeasts were identified at the species level by conventional morphological and biochemical methods and stored at −70°C in 10% glycerol. Before the initiation of the study, yeast isolates were subcultured on antimicrobial agent-free medium to ensure viability and purity.

TABLE 1. Caspofungin and amphotericin B MICs of 28 isolates of *Candida* spp. utilized in this study

Drug	*Candida* species[a]	Geometric mean MIC (range) (μg/ml) obtained by:	
		Broth dilution	Etest
Caspofungin	CA	0.03 (0.03)	0.02 (0.002–0.125)
	CP	0.85 (0.06–4.0)[b]	0.43 (0.125–2.0)[b]
	CG	2.0 (1.0–8.0)[b,c]	3.26 (0.25–16)[b,c]
Amphotericin B	CA	1.15 (1.0–2.0)	0.04 (0.002–0.125)
	CP	0.69 (0.25–1.0)	0.08 (0.002–1.0)
	CG	0.66 (0.25–2.0)	0.14 (0.01–2.0)

[a] Five isolates of *Candida albicans* (CA), 10 isolates of *C. guilliermondii* (CG), and 13 isolates of *C. parapsilosis* (CP) were tested. Each isolate was tested from two to four times by each method.

[b] MICs were significantly higher than those reported for CA (P values ranging from <0.001 to 0.002).

[c] MICs were significantly higher than those reported for CP ($P = 0.03$ [broth dilution] and $P < 0.001$ [E test]).

TABLE 2. Caspofungin and amphotericin B susceptibilities of seven isolates utilized in killing experiments and in mice

Strain or isolate[a]	Median MIC (range) (μg/ml) for:	
	Caspofungin	Amphotericin B
C. albicans SC5314	0.03 (0.03–1.0)	1.0 (0.50–1.0)
C. guilliermondii		
Isolate 1	2.0 (2.0–4.0)	0.25 (0.25–1.0)
Isolate 2	2.0 (1.0–2.0)	0.25 (0.25–0.5)
Isolate 3	8.0 (2.0–8.0)	0.25 (0.25–1.0)
C. parapsilosis		
Isolate 1	1.0 (0.25–2.0)	0.5 (0.25–1.0)
Isolate 2	0.5 (0.5–1.0)	1.0 (0.25–1.0)
Isolate 3	4.0 (2.0–4.0)	1.0 (0.25–1.0)

[a] Each isolate was tested seven times.

Drugs. CAS was used as commercial preparation (Cancidas; Merck Sharp & Dohme) for either in vitro or in vivo experiments. It was dissolved in sterile distilled water and in sterile saline solution for in vitro and in vivo studies, respectively. The pure powder (Sigma) of AMB was used for in vitro studies, and the commercial preparation (Fungizone; Bristol-Myers Squibb) was used for in vivo studies. It was dissolved in dimethyl sulfoxide and in sterile saline solution for in vitro and in vivo studies, respectively.

In vitro studies. (i) MICs. CAS and AMB MICs were determined either by the broth dilution method following the instructions established by the Clinical Laboratory Standards Institute (CLSI, formerly NCCLS) or by the Etest method (AB Biodisk, Skolne, Sweden) performed according to the manufacturer's instructions. Both tests were performed in RPMI 1640 medium buffered with morpholinepropanesulfonic acid (MOPS) buffer. For broth dilution method, both drugs were used at concentrations ranging from 0.03 to 64 μg/ml. CAS MICs were read at 24 h and considered the lowest drug concentration causing a significant reduction of growth below control growth levels. Each isolate was tested from two to seven times by both methods (13, 14).

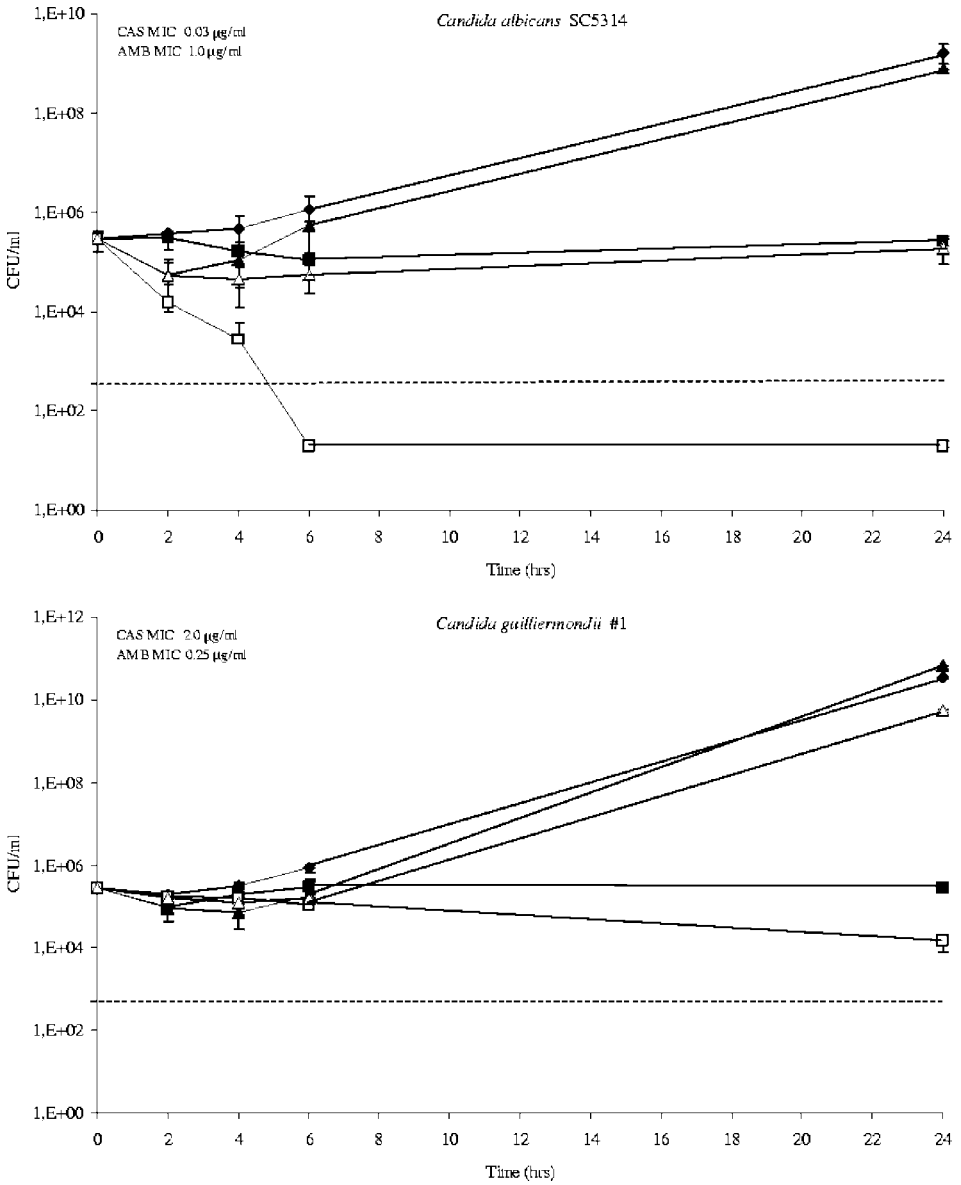


FIG. 1. Time-kill studies conducted with *C. albicans* SC5314, *C. guilliermondii*(isolates 1, 2, and 3), and *C. parapsilosis* isolates. (isolates 1, 2, and 3). Black diamonds, controls; closed squares, 1× AMB MIC; open squares, 8× AMB MIC; closed triangles, 1× CAS MIC; open triangles, 8× CAS MIC. The dotted lines represent a ≥99.9% growth reduction compared with the initial inoculum size (fungicidal effect). The limit of detection is 20 CFU/ml. Each data point represents the mean ± standard deviation (error bar) for three independent experiments.

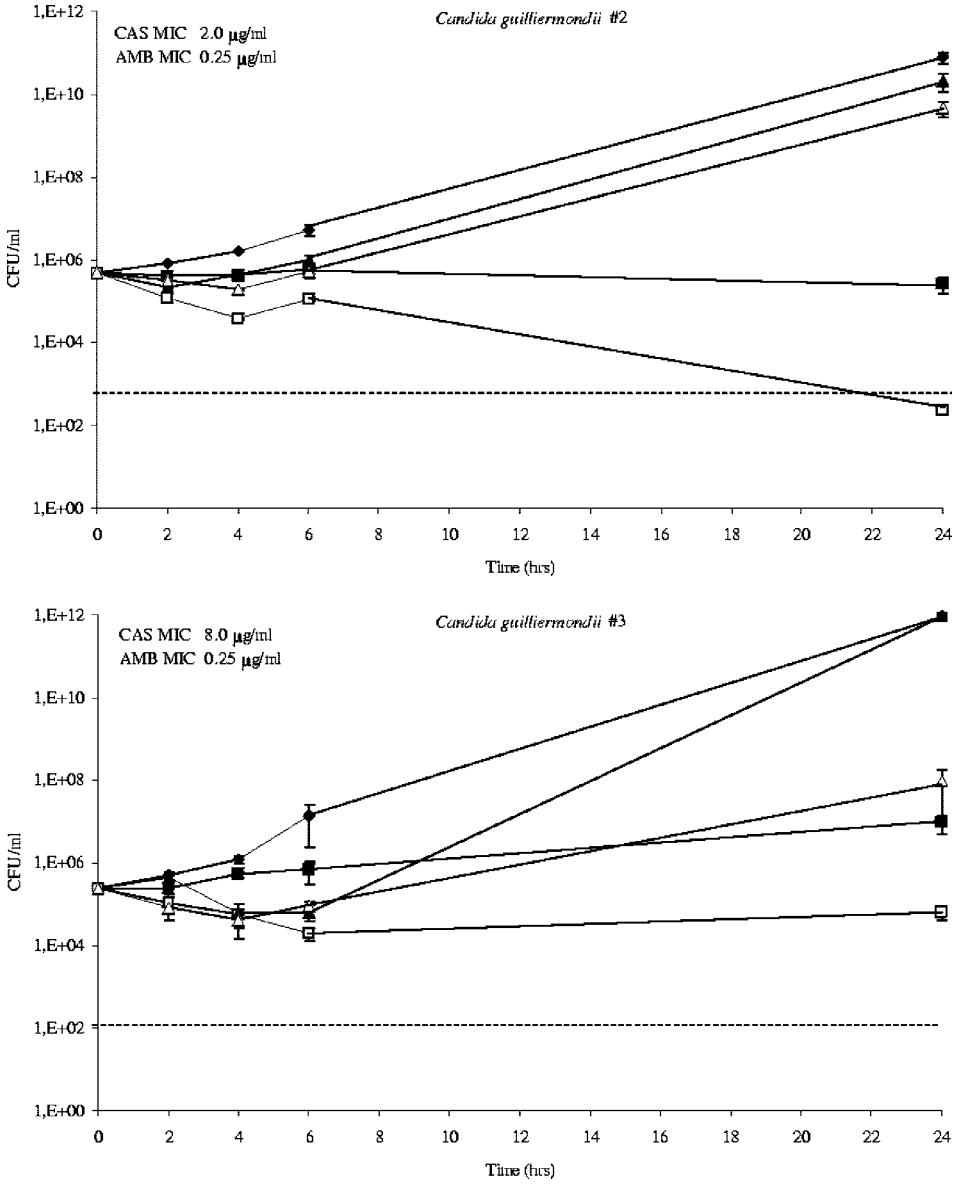
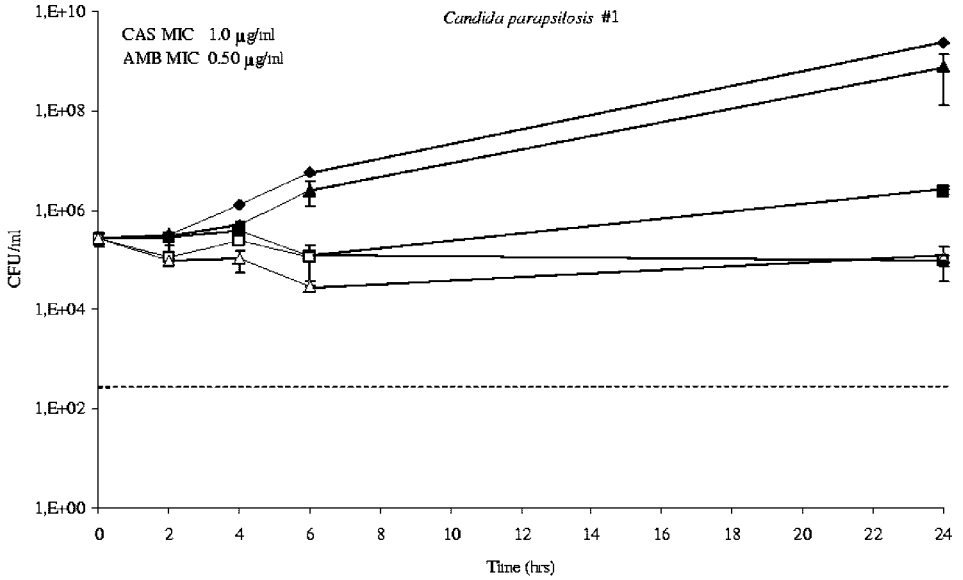
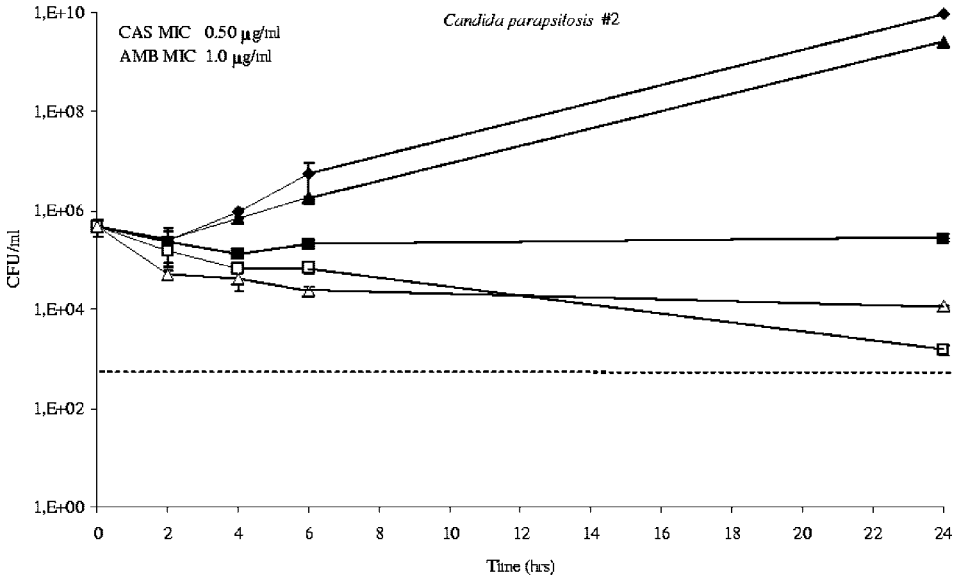


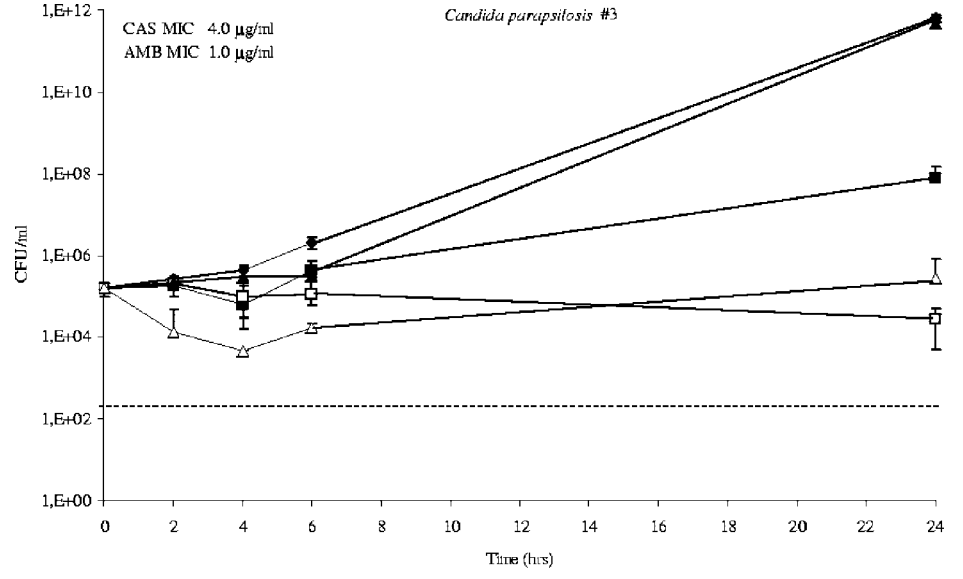
FIG. 1—*Continued.*

(ii) Killing curves. *C. albicans* SC5314 and three isolates each of *C. guilliermondii* (isolates 1, 2, and 3) and *C. parapsilosis* (isolates 1, 2, and 3) were used in killing experiments (19). Briefly, three to five colonies of each strain from a 24-h growth plate were suspended in 10 ml of sterile distilled water, and the turbidity was adjusted using spectrophotometric methods to a 0.5 McFarland standard (approximately 1×10^6 to 5×10^6 CFU/ml). One milliliter of the adjusted fungal suspension was added to 9 ml of either RPMI 1640 medium buffered with MOPS buffer plus an appropriate amount of drug. Both CAS and AMB were used at 1 and 8 times the MIC obtained by the broth dilution method. CAS and AMB MICs for the selected isolates are reported below (see Table 2). Test solutions were placed on a shaker and incubated at 35°C. At time points 0, 2, 4, 6, and 24 h following the introduction of the test isolate into the system, 100-μl aliquots were removed from each test solution. After 10-fold serial dilutions, a 50-μl aliquot from each dilution was streaked in triplicate onto Sabouraud dextrose agar plates for colony count determination. Following incubation at 35°C for 48 h, the number of CFU on each plate was determined. The limit of detection was 20 CFU/ml. Fungicidal activity was considered to be achieved when the number of CFU per milliliter was reduced by ≥99.9% compared to the initial inoculum size (19). Experiments were performed in triplicate.

In vivo studies. *C. albicans* SC5314, *C. guilliermondii* (isolates 1, 2, and 3), and *C. parapsilosis* (isolates 1, 2, and 3) were used for in vivo experiments. CD1 male mice (Charles River, Calco, Lecco, Italy) weighing 25 g were utilized in all experiments. In studies involving isolates of *C. guilliermondii* and *C. parapsilosis*, the mice were rendered neutropenic by intraperitoneal administration of cyclophosphamide (200 mg/kg of body weight/day) on days −4, +1, and +4 postinfection. Mice were infected intravenously with a yeast inoculum given in a 0.2-ml volume. Inoculum sizes were as follow: 2.2×10^5 CFU/mouse for *C. albicans* SC5314; 5.0×10^8 CFU/mouse, 8.5×10^8 CFU/mouse, and 1×10^9 CFU/mouse for *C. guilliermondii* isolates 1, 2, and 3, respectively; and 6.0×10^7 CFU/mouse, 3.4×10^6 CFU/mouse, and 3.5×10^8 CFU/mouse for *C. parapsilosis* isolates 1, 2, and 3, respectively. Both drugs were administered intraperitoneally for 4 consecutive days in a 0.2-ml volume starting 24 h postchallenge. CAS was given at 0.25, 1, and 5 mg/kg/day, while AMB was given at 1 mg/kg/day. Drug efficacy was assessed by determining the number of CFU per kidney pair. Briefly, the mice were sacrificed, the kidneys were homogenized, and diluted or undiluted aliquots, including the entire organ, were grown on Sabouraud dextrose agar for colony count determination. Tissue burden experiments were performed 24 h after the last dose (day 5 postinfection). There were seven or eight animals in







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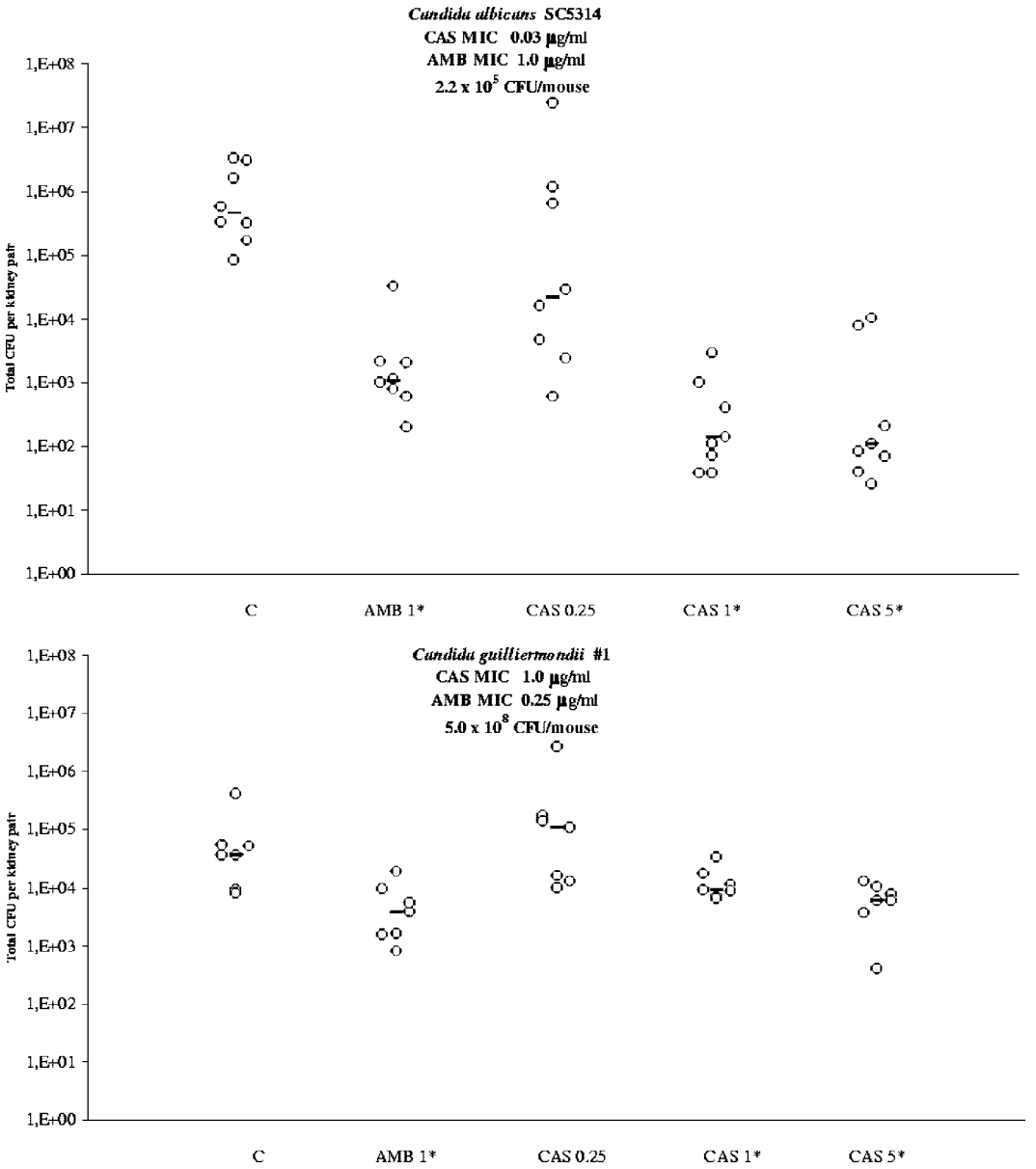

FIG. 2. Kidney fungal burden of CD1 mice. In studies involving isolates of *C. guilliermondii* and *C. parapsilosis*, the mice were rendered neutropenic. Animals were treated daily for 4 consecutive days with AMB at 1 mg/kg/day (AMB 1) and CAS at 0.25, 1, or 5 mg/kg/day (CAS 0.25, CAS 1, and CAS 5, respectively). Tissue burden experiments were performed on day 5 postinfection. The bars represent the medians. The asterisks at the bottom of panels indicate that the values were significantly different (P values of <0.05) from the values for the controls (C).

each control and treatment group. Animal experiments were conducted with the approval of the University of Ancona Ethics Committee.

Statistical analysis. The Mann-Whitney U test was used to compare either MICs or tissue burden counts. A P value of <0.05 was considered statistically significant.

RESULTS

CAS median MICs obtained by the broth dilution were 0.03 μg/ml for *C. albicans* ATCC 90029 and *C. albicans* SC5314 and 0.06 μg/ml for *C. parapsilosis* ATCC 22019. AMB median MICs obtained by the same method were 1.0 μg/ml for all three isolates.

CAS median MICs obtained by the Etest were 0.01 μg/ml, 0.06 μg/ml, and 0.125 μg/ml for *C. albicans* ATCC 90029, *C. albicans* SC5314, and *C. parapsilosis* ATCC 22019, respectively. AMB median MICs obtained by the Etest were 0.125 μg/ml for both ATCC isolates, while it was 0.06 μg/ml for *C. albicans* SC5314.

The overall susceptibilities of all 28 isolates tested are reported in Table 1.

Isolates of *C. albicans* were shown to be significantly more susceptible to CAS than isolates of *C. guilliermondii* or *C. parapsilosis*. Furthermore, isolates of *C. parapsilosis* were more susceptible to CAS than isolates of *C. guilliermondii* were. Both the broth microdilution and Etest methods confirmed this trend of susceptibility. AMB MICs did not significantly differ among isolates belonging to the three species of *Candida*.

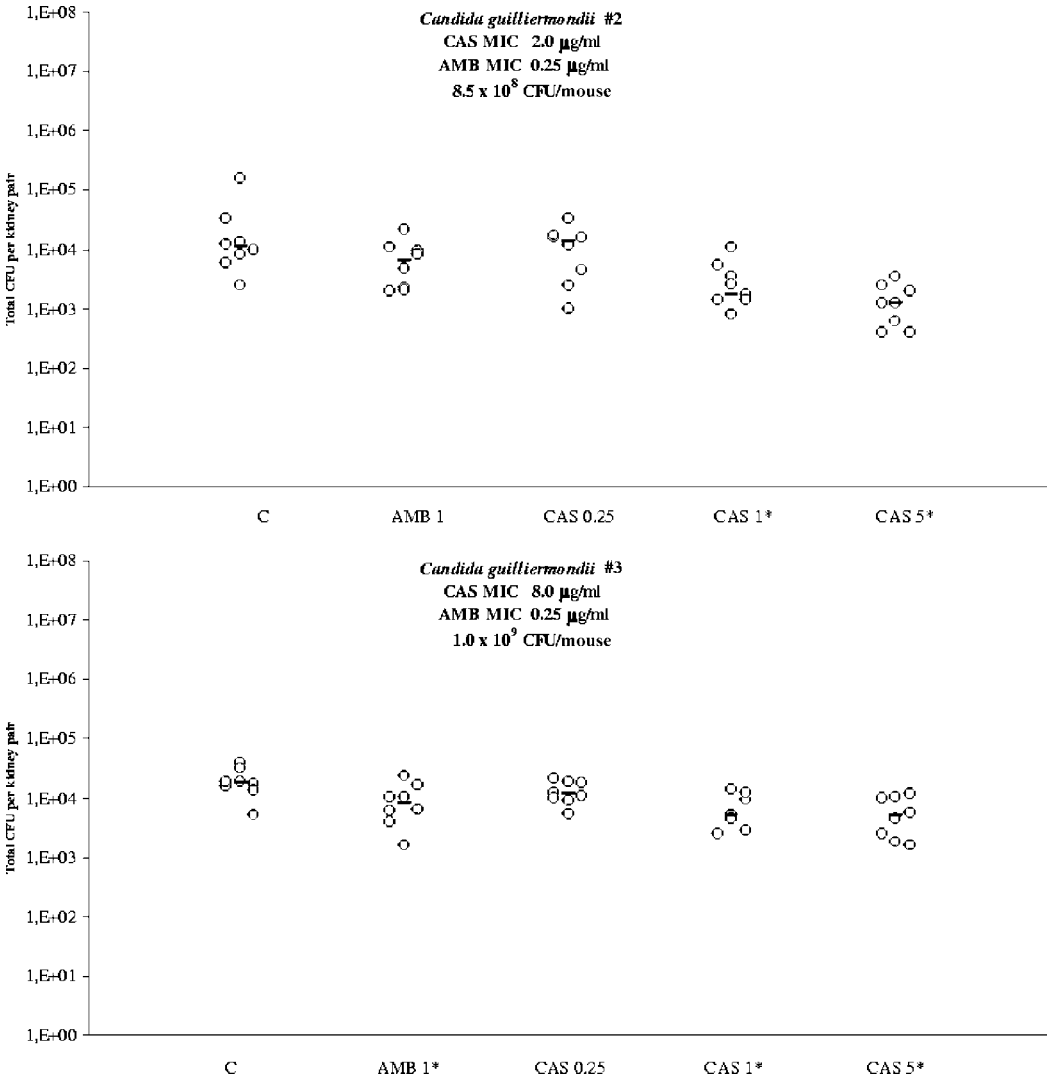

FIG. 2—*Continued.*

Then, we selected seven isolates for killing experiments (Table 2). Killing curves of the seven isolates tested are reported in Fig. 1.

AMB at the MIC was fungistatic against all seven isolates. AMB at eight times the MIC exerted a fungicidal activity against *C. albicans* SC5314 and *C. guilliermondii* isolate 2. This effect was reached upon approximately 6 and 22 h of incubation, respectively. The polyene, at the highest concentration, was fungistatic for the remaining five isolates.

CAS at the MIC exerted fungistatic activity only for 4 to 6 h of incubation against all isolates; afterwards, growth similar to that of the controls was often observed. The same phenomenon was seen in all isolates of *C. guilliermondii*, even when CAS was utilized at eight times the MIC, while at this concentration, the drug maintained fungistatic activity upon 24 h of incubation against *C. albicans* SC5314 and all isolates of *C. parapsilosis*.

To see whether the strains grown at the highest drug concentrations upon 24 h of incubation represent CAS- or AMB-resistant mutants, two single colonies from each strain/drug combination were randomly selected and tested by the broth dilution method. All strains tested maintained a susceptibility pattern similar (within a double dilution) to that of their respective parent isolates for both drugs (data no shown).

The results of in vivo studies are reported in Fig. 2.

In mice infected with *C. albicans* SC5314, all treatments, with the exception of CAS at 0.25 mg/kg/day, were effective at reducing the counts against the controls ($P < 0.001$).

Similarly, in mice infected with *C. guilliermondii* isolates 1 and 3, all treatments, with the exception of the lowest dose of CAS, were effective (P ranging from <0.001 to 0.04). Only CAS at 1 mg/kg/day ($P = 0.008$) and 5 mg/kg/day ($P = 0.001$) significantly reduced the counts with respect to the counts of controls in mice infected with *C. guilliermondii* isolate 2.

In mice infected with *C. parapsilosis* isolates 1 and 2, only AMB at 1 mg/kg/day and CAS at 5 mg/kg/day were effective at reducing fungal burden with respect to the control (P ranging from <0.001 to 0.006). Finally, in mice infected with *C. parapsilosis* isolate 3, not only AMB at 5 mg/kg/day and CAS at 5 mg/kg/day but also CAS at 1 mg/kg/day reduced the counts in

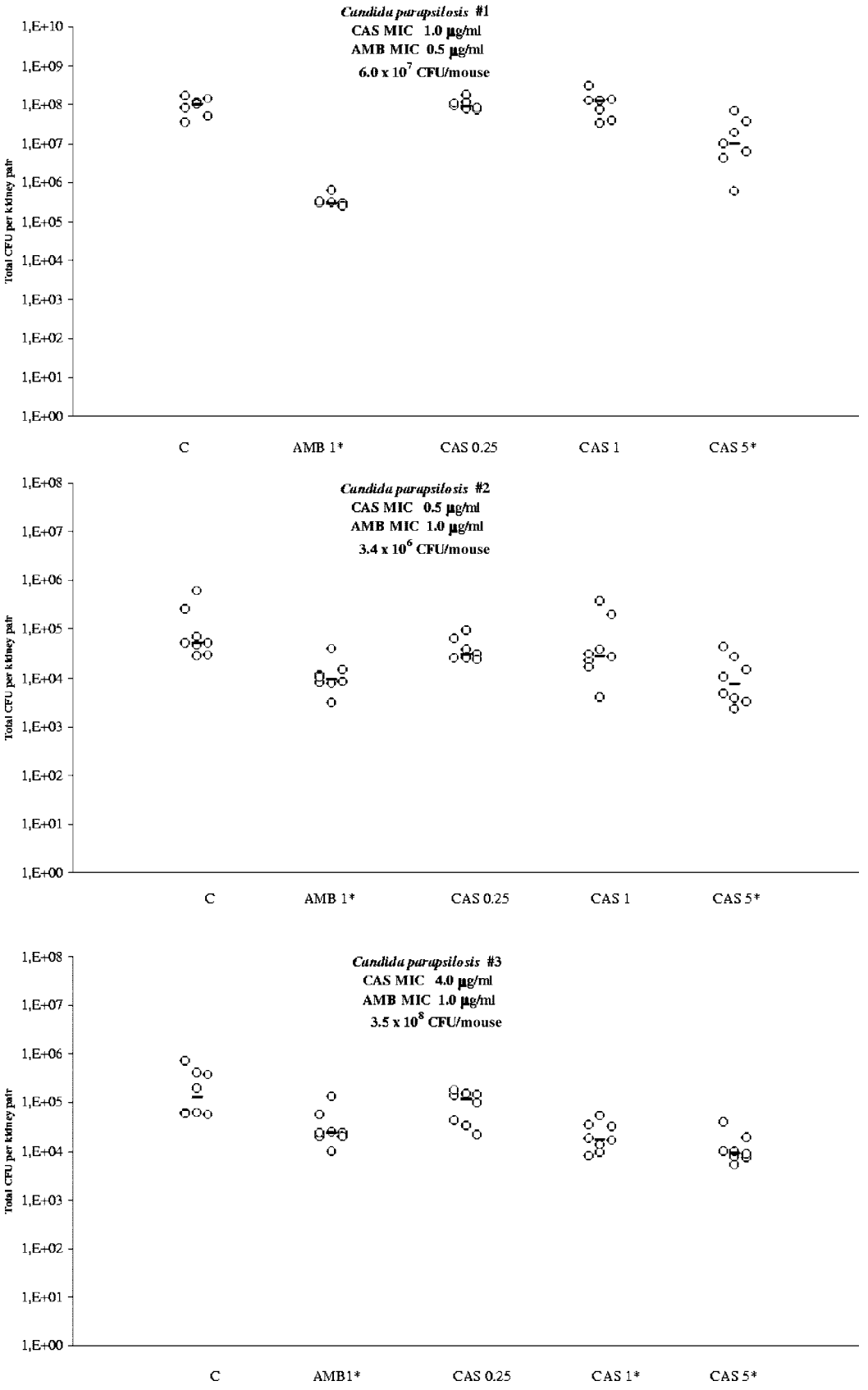

FIG. 2—*Continued.*

the kidney compared with the controls ($P = 0.004$ for AMB and $P < 0.001$ for both CAS doses).

DISCUSSION

In this study, we analyzed the in vitro and in vivo activities of CAS against *C. guilliermondii* and *C. parapsilosis*. Although we utilized only 5 to 13 strains of each species, we confirmed that *C. guilliermondii* and *C. parapsilosis* isolates are less susceptible in vitro to CAS than *C. albicans* is. This was demonstrated by the broth dilution method and confirmed by the Etest. Our MIC data, which are in agreement with those previously reported by others (7, 18), clearly showed a precise rank order of susceptibility to the echinocandin compound with *C. albicans* > *C. parapsilosis* > *C. guilliermondii*. These findings partially mirrored those obtained by killing experiments. Although CAS did not exert a fungicidal activity against any of the strains tested, at the highest concentration, after 24 h it reduced the CFU of *C. albicans* SC5314 and *C. parapsilosis* isolates from 0.2 to 1.6 $\log_{10}$. It was completely ineffective (growth similar to that of control) against all isolates of *C. guilliermondii*. These data are different than those reported in a previous study (5) where it was shown that CAS at ≥4 times the MIC exerted a fungicidal activity against two isolates of *C. guilliermondii*. It must be noted, however, that the highest concentration of CAS tested in our study was 64 μg/ml (8 times the MIC), while those utilized in the study mentioned above were 512 μg/ml and 1,024 μg/ml (4 and 8 times the MIC, respectively). The reason why *C. guilliermondii* and *C. parapsilosis* are less susceptible in vitro to CAS than *C. albicans* is unknown at present. Either structural differences of the cell wall components, a reduced affinity for the glucan synthase protein complex, or a variation of its regulatory network might explain this considerable interspecies variation (4, 6). This trend of susceptibility is limited not only to CAS but also to any echinocandin derivative. Recently, Pfaller et al. tested up to 2,000 clinical isolates of *Candida* spp. to anidulafungin and found MIC_{90} of 2.0 μg/ml for both *C. parapsilosis* and *C. guilliermondii*, while *C. albicans* showed an MIC_{90} of 0.125 μg/ml (16). Similar results were reported for micafungin (7, 15).

Our in vivo studies showed that CAS is effective against *C. guilliermondii* and *C. parapsilosis*. To our knowledge, this is the first study in which an echinocandin derivative is utilized in an experimental model of infection due to *C. guilliermondii*. As far as we know, all models have been done with *C. albicans* (including fluconazole-resistant isolates), *Candida glabrata*, *Candida krusei*, *Candida tropicalis* and *C. parapsilosis* (1, 4, 8, 9).

Our results demonstrated that CAS at doses as low as 1 mg/kg/day was effective against all three isolates of *C. guilliermondii*.

Despite the fact that *C. parapsilosis* isolate 3 was shown to be less susceptible in vitro than *C. parasilosis* isolates 1 and 2 were to CAS, the mice infected with this isolate responded at doses as low as 1 mg/kg/day, while only the highest dose of CAS was effective against the other two isolates. This finding is difficult to explain and underlines the imperfect correlation between in vitro and in vivo results. Overall, our data on *C. parapsilosis* are in agreement with a previous study, which showed that CAS at 1.5 mg/kg/dose twice a day was effective at reducing the kidney burden of DBA/2N mice challenged with *C. parapsilosis* (1).

The reason why *C. parapsilosis* and *C. guilliermondii* isolates with higher CAS MICs responded in vivo to this drug as well as isolates with lower MICs did can be explained with data reported by Louie et al. (11). They found that CAS levels persisted in kidney tissue well after serum concentrations fall below the MIC and thus exerted a greater effect than might be expected.

Since echinocandins show a concentration-dependent activity in experimental models of infections due to *Candida* species other than *C. guilliermondii* and *C. parapsilosis* (19), further studies employing additional doses are needed to clarify this issue in these two species as well.

In conclusion, we demonstrated that CAS was active in experimental systemic candidiasis due to *C. guilliermondii* and *C. parapsilosis*, but this activity required relatively high drug dosages and the overall CFU reduction was approximately 100-fold less than that for *C. albicans*.

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