Killing Kinetics of Caspofungin, Micafungin, and Amphotericin B against *Candida guilliermondii*

Emilia Cantón,^{1*} Javier Pemán,² Macrina Sastre,¹ Mónica Romero,¹ and Ana Espinel-Ingroff,³

Unidad de Microbiología Experimental-Centro de Investigación¹ and Servicio de Microbiología,² Hospital Universitario La Fe, 46009 Valencia, Spain, and Division of Infectious Disease, VCU Medical Center, Richmond, Virginia 23298-0049³

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Amphotericin B (AMB), micafungin, and caspofungin MICs, minimal fungicidal concentrations, and time-killing curves were determined in the presence and absence of 10% inactivated serum. AMB was the only agent with consistent killing activity (time required to achieve 99.9% of growth reduction was 2.1 to 3.2 h). The presence of serum enhanced caspofungin activity but lowered those of micafungin and AMB.

Candida guilliermondii is infrequently isolated from blood cultures (1 to 5%) (14), and infections caused by this species have been reported in cancer, surgical, and intensive care unit patients (13–14), including a pseudo-outbreak of candidemia in a neonatal intensive care unit (23). Malignancy, neutropenia, and bone marrow transplantation have been reported as risk factors for acquiring *C. guilliermondii* infections (22). Treatment of these infections may present problems, especially for immunocompromised patients; a high percentage of strains have diminished susceptibility to fluconazole (MIC at which 90% of organisms are inhibited, 16 mg/liter) and itraconazole (MIC at which 90% of organisms are inhibited, 1 mg/liter) (17–18). Amphotericin B (AMB), caspofungin, and micafungin have fungicidal activity against some *Candida* spp. (3–4, 8–9), but most studies have focused on *Candida albicans* and little is known about the killing activity of these agents against *C. guilliermondii*. A previous evaluation demonstrated that the same AMB MIC may correspond to different killing activities, depending on the *Candida* species or strain tested, and that each species has a different AMB killing pattern (5). Because the killing kinetics of AMB, caspofungin, and micafungin against *C. guilliermondii* are unknown, this study aimed to characterize the in vitro pharmacodynamics and fungicidal activities of these drugs against this species. Since these agents are highly protein bound (>90%) (9, 10, 11), the influence of serum on their in vitro activity has also been evaluated. For the first time, we provide the dynamics of the fungicidal activity of these three agents against *C. guilliermondii*.

Drugs. AMB deoxycholate (Bristol-Myers Squibb, Madrid, Spain) was dissolved in dimethyl sulfoxide, and caspofungin (Merck Sharpe & Dome, Madrid, Spain) and micafungin (Fujisawa Pharmaceutical Company, Japan) were dissolved in water. Further drug dilutions were prepared in standard RPMI 1640 medium (Sigma-Aldrich, Madrid, Spain) as recommended in the M27-A2 document (16).

MIC and MFC determination. MICs were determined for 19 *C. guilliermondii* clinical isolates at least twice (on different

days) in RPMI and RPMI plus 10% inactivated fetal bovine serum (FBS) (Sigma-Aldrich) by the M27-A2 method but using a higher inoculum (2.5×10^4 CFU/ml). Complement was inactivated by heating the undiluted serum (30 min, 60°C). Both MIC₂ and MIC₀ ($\geq 50\%$ and 100% growth reduction, respectively) were determined for caspofungin and micafungin and MIC₀s for AMB. Minimal fungicidal concentrations (MFCs) were obtained by transferring 0.1 ml from all clear MIC wells (with or without serum) onto Sabouraud dextrose agar plates. The MFC was the lowest concentration that killed $\geq 99.9\%$ of cells (< 5 colonies) (4). *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6452 isolates were included as controls. AMB and caspofungin MICs for these two strains were within the accepted limits (2, 16).

Time-killing studies. As previously described (5), tests were conducted for 8 of the 19 isolates (in duplicate on 2 separate days) with RPMI or RPMI plus 10% FBS inactivated, 10^5 -CFU/ml inocula with 5-ml volumes and a 0.12-, 0.5-, 2-, 8-, or 32-mg/liter drug concentration. CFU were determined at 0, 2, 4, 6, 24, and 48 h. MICs and MFCs of the three agents were determined simultaneously, under the same testing conditions each time. Drugs were treated with the same heated serum each time a set of strains was tested in the presence or absence of serum. Furthermore, prepared microplates containing serum and drug were not stored before testing.

Data analysis. For calculations of geometric mean MICs (GM-MICs) and GM-MFCs, off-scale MICs and MFCs were converted to the next highest values. Time-killing data were fitted to an exponential equation: $N_t = N_0 \times e^{-Kt}$ (N_t , viable cells at time t ; N_0 , starting inoculum; K , killing rate; t , incubation time). The time to achieve 50, 90, 99, or 99.9% growth reduction at each concentration tested was calculated from the K value as described elsewhere (5).

Our echinocandin MICs (Table 1) were in agreement with those previously reported for this species (3, 8, 15, 19, 20), where the MIC₂ was more reproducible than the MIC₀. AMB was the most active agent, followed by micafungin and caspofungin (GM-MIC₀ values, 0.1, 0.9, and 5.1 $\mu\text{g/ml}$, respectively). The antifungal activity in the presence of inactivated serum was isolate dependent, where AMB and micafungin lost activity (overall GM-MIC₀ increases of 3.14 times for AMB and 3.88 times for micafungin); in contrast,

* Corresponding author. Mailing address: Unidad de Microbiología Experimental, Centro de Investigación, Hospital Universitario La Fe, Avenida Campanar 21, 46009 Valencia, Spain. Phone: 34 96 1973111. Fax: 34 96 3868718. E-mail: canton_emi@gva.es.

TABLE 1. Activities of amphotericin B, caspofungin, and micafungin against 19 isolates of *C. guilliermondii*^a

Strain (no. of times tested) or parameter	Medium	Activity of drug							
		Amphotericin B		Caspofungin			Micafungin		
		MIC ₀	MFC	MIC ₂	MIC ₀	MFC	MIC ₂	MIC ₀	MFC
CJ-12 (4)	RPMI	0.12–0.5	0.25–0.5	1–2	2–>8	>8	0.25–1	0.5–2	0.5–8
	RPMI+S	0.12–1	0.25–1	0.5–1	2–>8	>8	0.5–4	2–8	4–>8
CJ-19 (4)	RPMI	0.12–0.5	0.12–0.5	1–2	1–2	1–>8	0.12–0.5	0.12–0.5	0.25–0.5
	RPMI+S	0.25–1	0.5–1	0.06–0.5	0.12–0.5	0.12–1	1–4	2–4	2–4
CJ-21 (5)	RPMI	0.016–0.06	0.03–0.5	1	1–>8	>8	0.12	0.12–0.5	0.25–1
	RPMI+S	0.03–0.25	0.03–0.5	0.06–2	0.12–>8	>8	1–2	2–4	4–8
CJ-23 (5)	RPMI	0.12–0.5	0.25–0.5	1–2	2–>8	2–>8	0.12–0.25	0.12–4	0.12–>8
	RPMI+S	0.06–1	0.25–2	0.03–2	0.25–>8	0.5–>8	1–4	1–8	1–>8
CJ-24 (5)	RPMI	0.03–0.12	0.06–0.12	1	1–>8	>8	0.12–0.25	0.12–2	0.25–>8
	RPMI+S	0.06–0.25	0.25–1	0.06–2	0.25–>8	>8	1–2	2–4	4–>8
CJ-26 (3)	RPMI	0.03–0.12	0.03–0.25	0.5–1	>8	>8	0.008–0.25	0.25–1	1–8
	RPMI+S	0.25–1	0.5–2	0.03–0.25	0.06–0.5	>8	0.5–2	1–4	4–>8
CJ-46 (3)	RPMI	0.06–0.25	0.06–0.5	1–2	1–2	2–>8	0.25–0.5	0.5–1	1–4
	RPMI+S	0.25–1	0.25–1	0.12–1	0.25–1	0.5–8	2–4	2–8	2–8
CY-115 (2)	RPMI	0.12	0.12	0.5–1	>8	>8	0.12–0.5	4–>8	>8
	RPMI+S	0.25	0.25	0.12–2	>8	>8	1–2	2–>8	>8
CY-116 (2)	RPMI	0.12	0.12	1	>8	>8	0.5	1	4
	RPMI+S	0.25	0.25	2	>8	>8	2–4	4–8	8
CY-131 (2)	RPMI	0.12	0.12	1	4	>8	0.06–0.25	0.25–0.5	0.5
	RPMI+S	0.12	0.25	0.5	4	>8	0.5–2	2–8	8
CY-132 (2)	RPMI	0.12	0.25	1	1	>8	0.12	0.25–0.5	1
	RPMI+S	0.25	0.25	0.12–0.5	0.5	>8	2	2–4	8
CY-133 (2)	RPMI	0.12	0.12	0.5–1	1–>8	>8	0.12–0.25	0.5–2	1
	RPMI+S	0.25	0.25	0.12–2	0.25–>8	>8	1	4	8
CY-134 (2)	RPMI	0.06	0.06	0.12–0.25	0.5–1	1	0.008–0.06	0.25	0.5
	RPMI+S	0.12	0.12	0.12–0.25	0.12–0.5	0.5	0.5–1	1–4	>8
CY-135 (2)	RPMI	0.12	0.12	1	>8	>8	0.12–1	8–>8	>8
	RPMI+S	0.25	0.5	0.25–1	>8	>8	2	>8	>8
CY-136 (2)	RPMI	0.5	0.5	1	>8	>8	0.5	2–>8	>8
	RPMI+S	0.5	0.5	0.5	>8	>8	0.5	2–>8	>8
CY-137 (2)	RPMI	0.25	0.25	1	>8	>8	0.5	1–2	4
	RPMI+S	0.25	0.25	1	>8	>8	2–4	4–8	>8
CY-138 (2)	RPMI	0.12	0.12	1	>8	>8	0.5	>8	>8
	RPMI+S	0.5	0.5	0.5–1	>8	>8	2	4–>8	>8
CY-139 (2)	RPMI	0.25	0.5	1	>8	>8	0.25–0.5	4–>8	>8
	RPMI+S	0.5	0.5	2	>8	>8	2–4	4–>8	>8
CY-140 (2)	RPMI	0.12	0.25	0.25–1	1–>8	>8	0.03–0.06	0.03–0.06	0.03–0.06
	RPMI+S	0.25	0.5	0.25–1	0.25–1	>8	0.25–0.5	0.5	0.5
ATCC 6458 (3)	RPMI	0.5	0.5	1	1–2	1–2	0.06–0.12	0.06–0.12	0.12
	RPMI+S	1	2	0.12–1	1	1	1	1	1
ATCC 22019 (4)	RPMI	0.5–1	1	1–2	2–>8	2–>8	0.5–1	0.5–1	0.5–1
	RPMI+S	1	1–2	0.5–1	1–2	2–>8	2–4	2–4	4–8
Overall range	RPMI	0.016–0.5	0.03–0.5	0.12–2	0.5–>8	1–>8	0.008–1	0.03–>8	0.03–>8
	RPMI+S	0.06–1	0.06–2	0.03–2	0.06–>8	0.12–>8	0.25–4	0.5–>8	0.5–>8
Overall MIC ₅₀	RPMI	0.12	0.12	1	>8	>8	0.25	1	1
	RPMI+S	0.25	0.5	0.5	1	>8	2	4	8
Overall MIC ₉₀	RPMI	0.5	0.5	2	>8	>8	0.5	>8	>8
	RPMI+S	1	1	2	>8	>8	4	>8	>8
Overall GM	RPMI	0.108	0.153	0.971	5.087	9.514	0.193	0.905	1.649
	RPMI+S	0.34	0.499	0.434	1.727	5.652	1.587	3.512	6.579

^a RPMI+S and RPMI, RPMI in the presence or absence of 10% inactivated FBS, respectively; MIC₂ and MIC₀, concentrations at which 50 and 100% of growth is inhibited, respectively; MIC₅₀ and MIC₉₀, concentrations at which 50% and 90% of organisms are inhibited, respectively; GM, geometric mean.

caspofungin activity was slightly higher (overall 0.3-times-lower GM-MIC₀) (Table 1). The lack of data regarding the effect of serum on the antifungal activities of these agents against *C. guilliermondii* precluded comparisons with our results. However, the effect has been antifungal agent dependent and controversial for *C. albicans* and *Aspergillus fumigatus*. Although micafungin MIC increases (three to seven dilutions, species dependent) were reported (9, 20) in

the presence of either inactivated or noninactivated serum for *C. albicans*, the presence of serum did not affect AMB activity for this species in another study (24). Both no effect (*C. albicans*) and an increased activity (*A. fumigatus*) have been reported with caspofungin (3, 6). The reduction of the in vitro activities of AMB and micafungin against *C. guilliermondii* may be due to their high level of protein binding (>90%), while the increased activity of caspofungin in the

FIG. 1. Time-killing kinetics assays of AMB, micafungin, and caspofungin against *C. guilliermondii*. Average datum points and standard deviations are provided for eight *C. guilliermondii* isolates. Upper panels, RPMI medium. Lower panels, RPMI plus 10% inactivated FBS. A, amphotericin B; B, micafungin; C, caspofungin.

presence of inactivated serum indicated that this increase was not due to complement mechanisms.

Figure 1 depicts the killing activity for *C. guilliermondii* (mean and standard deviation) in the presence or absence of serum. The killing activities of caspofungin and micafungin were not sustained because cell growth was seen at 24 h. In contrast, the killing kinetics of AMB was fast and increased with the drug concentration. The presence of serum diminished the killing activity of AMB (K value 1.8 times lower in presence of serum, respectively). The relationship between drug concentration and lethality was lineal (Fig. 2). The times required for 2 mg/liter of AMB to kill 50%, 90%, 99%, and 99.9% of the initial inoculum ranged from 12 to 19 min (t_{50} range) to 2.1 to 3.2 h ($t_{99.9}$ range) (Table 2).

Killing patterns of these three antifungal agents have been reported for *C. albicans*, *Candida glabrata*, *C. krusei*, *C. parapsilosis*, and *Candida tropicalis* (3, 5, 9); we extended this knowledge for *C. guilliermondii*. The clinical response to AMB therapy for *C. guilliermondii* infections is controversial. Treatment failures have been documented in invasive or ocular infections (1, 7, 12, 21, 22), and they have been associated with either potential in vitro resistance (MICs of 1 to 4 mg/liter) or neutropenia. We did not detect in vitro resistance to AMB, since all isolates evaluated were killed by ≤ 2 µg/ml of AMB in less than 24 h ($t_{99.9} = 2.68 \pm 0.48$ h) in the presence or absence of heated serum.

In summary, for the first time this study has provided the dynamics of the fungicidal activities of AMB, caspofungin, and micafungin against *C. guilliermondii*. AMB, caspofun-

FIG. 2. Relationship of amphotericin B, caspofungin, and micafungin concentrations and K values (regression lines) of survival time for *C. guilliermondii* in the presence (continuous line) or absence (dotted line) of inactivated fetal (10%) bovine serum. For caspofungin and micafungin, regression lines are practically horizontal (with and without serum) and almost identical (lines overlap).

TABLE 2. Times to achieve 50, 90, 99, and 99.9% reductions in growth by amphotericin B for *C. guilliermondii* from the starting inoculum

Parameter	Mean value (h) $\pm$ SD (range) at amphotericin B concn (μ g/ml) of:					
	0.12	0.5	1	2	8	32
t_{50}	NR ^a	NR	1.74 $\pm$ 2.17 (0.21–3.27)	0.29 $\pm$ 0.05 (0.21–0.32)	0.19 $\pm$ 0.05 (0.10–0.22)	0.27 $\pm$ 0.25 (0.10–0.71)
t_{90}	NR	NR	4.44 $\pm$ 7.21 (0.21–10.89)	0.83 $\pm$ 0.16 (0.69–1.08)	0.54 $\pm$ 0.16 (0.34–0.75)	0.63 $\pm$ 0.44 (0.34–1.43)
t_{99}	NR	NR	9.21 $\pm$ 14.41 (1.39–21.78)	1.72 $\pm$ 0.32 (1.4–2.16)	1.11 $\pm$ 0.33 (0.69–1.5)	1.13 $\pm$ 0.61 (0.69–2.15)
$t_{99.9}$	NR	NR	14.66 $\pm$ 21.62 (2.1–32.67)	2.68 $\pm$ 0.48 (2.09–3.24)	1.73 $\pm$ 0.49 (1.05–2.26)	1.53 $\pm$ 0.60 (1.04–2.26)

^a NR, not reached.

gin, and micafungin were active against *C. guilliermondii*, but only AMB showed fungicidal activity in the absence or presence of serum.

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