Species-Specific and Drug-Specific Differences in Susceptibility of *Candida* Biofilms to Echinocandins: Characterization of Less Common Bloodstream Isolates

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Candida species other than *Candida albicans* are increasingly recognized as causes of biofilm-associated infections. This is a comprehensive study that compared the *in vitro* activities of all three echinocandins against biofilms formed by different common and infrequently identified *Candida* isolates. We determined the activities of anidulafungin (ANID), caspofungin (CAS), and micafungin (MFG) against planktonic cells and biofilms of bloodstream isolates of *C. albicans* (15 strains), *Candida parapsilosis* (6 strains), *Candida lusitaniae* (16 strains), *Candida guilliermondii* (5 strains), and *Candida krusei* (12 strains) by XTT [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] assay. Planktonic and biofilm MICs were defined as ≥50% fungal damage. Planktonic cells of all *Candida* species were susceptible to the three echinocandins, with MICs of ≤1 mg/liter. By comparison, differences in the MIC profiles of biofilms in response to echinocandins existed among the *Candida* species. Thus, *C. lusitaniae* and *C. guilliermondii* biofilms were highly recalcitrant to all echinocandins, with MICs of ≥32 mg/liter. In contrast, the MICs of all three echinocandins for *C. albicans* and *C. krusei* biofilms were relatively low (MICs ≤ 1 mg/liter). While echinocandins exhibited generally high MICs against *C. parapsilosis* biofilms, MFG exhibited the lowest MICs against these isolates (4 mg/liter). A paradoxical growth effect was observed with CAS concentrations ranging from 8 to 64 mg/liter against *C. albicans* and *C. parapsilosis* biofilms but not against *C. krusei*, *C. lusitaniae*, or *C. guilliermondii*. While non-*albicans Candida* planktonic cells were susceptible to all echinocandins, there were drug- and species-specific differences in susceptibility among biofilms of the various *Candida* species, with *C. lusitaniae* and *C. guilliermondii* exhibiting profiles of high MICs of the three echinocandins.

*C*andida biofilms have been associated with many infections, including catheter-related fungemia, endocarditis, bone and joint infections, and others, with high morbidity and considerable mortality (1–3). In addition, oropharyngeal and vaginal *in vivo* models have demonstrated that mucosal *Candida* infections are due to the development of biofilms, a process controlled by morphogenetic and biofilm regulators (4, 5). Mature *Candida* biofilms consist of a complex 3-dimensional structure of fungal cells held together by pseudohyphae and a carbohydrate-rich extracellular matrix (6, 7).

While *Candida albicans* remains the predominant fungus responsible for bloodstream infections, in recent years there has been a trend of an increased incidence of candidemia caused by non-*albicans Candida* species. Overall, the most common non-*albicans Candida* sp. isolated from blood cultures is *Candida parapsilosis*; however, other *Candida* spp., including *Candida krusei*, *Candida lusitaniae*, and *Candida guilliermondii*, also cause candidemia (8–10). The biofilm-forming capacity of non-*albicans Candida* spp. has been implicated as a potential virulence factor in the development of candidemia for patients with vascular catheters, as the latter provide a suitable environment in which fungal organisms may grow and from which they may detach and colonize other body sites through the bloodstream (11, 12). In addition to vascular catheters, *Candida* spp. can cause difficult-to-treat infections in association with other inserted foreign bodies.

Echinocandins target (1→3)-β-D-glucan synthase, an enzyme absent in mammalian cells but essential for cell wall structural integrity and the function of many pathogenic fungi. Echinocandins are fungicidal for *Candida* planktonic cells (nonattached cells) and exhibit activity against mature biofilms of several *Candida* species (13–17). There have been several studies on the efficacy of echinocandins against *Candida* biofilms (18–21), the occurrence of the paradoxical effect observed with this class of antifungals (22, 23), and the role of echinocandin lock solutions in the management of candidiasis related to implanted devices (24); however, there are no comprehensive reports that have compared the *in vitro* activities of the three echinocandins against biofilms formed by different non-*albicans Candida* spp.

The aim of this study was to determine the antifungal activities of anidulafungin (ANID), caspofungin (CAS), and micafungin (MFG) against planktonic cells as well as biofilms formed by non-*C. albicans Candida* bloodstream isolates, such as *C. parapsi-*

TABLE 1 MICs of echinocandins for planktonically grown cells and biofilms of different bloodstream *Candida* isolates determined by the XTT assay

Species (n = 54)	Mean (range) MIC (mg/liter)					
	ANID		CAS		MFG	
	Planktonic cells	Biofilms	Planktonic cells	Biofilms	Planktonic cells	Biofilms
C. albicans (n = 15)	≤0.007	0.06[a] (0.015–0.06)	0.5 (0.25–0.5)	0.5 (0.25–4)	≤0.06	0.25[a] (0.06–2)
C. parapsilosis (n = 6)	0.06 (0.03–0.125)	32[a,b] (2–64)	1 (0.25–1)	64[a,b] (2–128)	0.5 (0.06–0.5)	4[a] (2–8)
C. krusei (n = 12)	0.125 (0.06–0.125)	0.125[b] (0.06–0.125)	1 (0.5–1)	1[b] (0.5–1)	≤0.06	0.125[a,b] (0.06–0.25)
C. lusitaniae (n = 16)	0.125 (0.03–0.125)	>256[a]	1 (0.25–1)	64[a] (16–64)	≤0.06	>256[a]
C. guilliermondii (n = 5)	1 (0.5–1)	32[a] (8–128)	1 (0.5–1)	64[a] (32–64)	0.125 (0.06–0.125)	>256[a]

[a] Significant differences between MICs of echinocandins against planktonic cells versus biofilms of *Candida* spp. (*P* < 0.01). These values have been derived from data presented in detail in Fig. 1 to 5.
[b] Significant differences between MICs of echinocandins against biofilms within the same species.

losis, *C. krusei*, *C. lusitaniae*, and *C. guilliermondii*, in comparison to those of *C. albicans*.

(This study was presented in part at the 52nd Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco, CA, 9 to 12 September 2012.)

MATERIALS AND METHODS

Clinical isolates and growth conditions. A total of 54 bloodstream isolates of *C. albicans* and non-*albicans Candida* species recovered from pediatric and adult immunocompromised and critically ill patients were studied. Twenty-six isolates were obtained from the Laboratory of Infectious Diseases Collection, Aristotle University, Hippokration Hospital, and were isolated at the Hippokration Hospital of Thessaloniki between October 1996 and June 2007, and 28 were obtained from the UOA/HCPF929 Collection, University of Athens, and were isolated between January 2010 and January 2011. Of the non-*albicans Candida* species isolated, *C. parapsilosis* was one of the most frequently identified non-*C. albicans Candida* isolates, *C. krusei* was a less frequently identified but azole-resistant species, and *C. lusitaniae* and *C. guilliermondii* were uncommonly reported bloodstream isolates (25–27). Species identification of these isolates was performed using a Vitek II system or an API ID32C kit (both from bioMérieux, Marcy l'Etoile, France) according to the manufacturer's instructions. The clinical isolates from the Hippokration collection were also identified by the germ tube test in serum, whereas the isolates obtained from University of Athens were identified by sequencing of the internal transcribed spacer (ITS) 1 and 2 noncoding ribosomal regions and by sequencing of the 26S ribosomal DNA gene, variable region D1/D2 (28, 29). Stocks were maintained at −80°C in yeast-peptone-dextrose broth with 10% to 25% glycerol (Oxoid, Cambridge, United Kingdom) solution. The clinical isolates were subcultured after overnight incubation at 37°C on Sabouraud (Scharlau Chemie, S.A., Barcelona, Spain) agar plates containing 0.05 mg/ml chloramphenicol and 0.25 mg/ml gentamicin. Two to three colonies from each isolate were subsequently transferred to 20 ml of yeast nitrogen base (Difco Laboratories, Detroit, MI) medium supplemented with 2% glucose and incubated at 37°C overnight on a rocking table. The grown cultures were harvested by centrifugation at 2,000 rpm for 10 min, washed twice with 10 ml of phosphate-buffered saline (PBS) solution (0.02 M phosphate, 0.15 M NaCl; pH 7.2), and resuspended in RPMI 1640 medium (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) buffered to pH 7.2 with 3-(*N*-morpholino)propanesulfonic acid (Sigma-Aldrich Chemie GmbH). For all experiments, the organisms were used at a final concentration of 10^6/ml.

Biofilm formation. Mature *Candida* biofilms were formed using 96-well microtiter polystyrene plates as previously described (30). Briefly, aliquots of 100 μl of yeast suspension were inoculated into each well and incubated for 48 h (*C. albicans*, *C. lusitaniae*, and *C. guilliermondii*) or 72 h (*C. parapsilosis* and *C. krusei*) at 37°C with shaking for the formation of comparable biofilms. Plates were then centrifuged at 4,000 rpm for 20 min and washed once with PBS to remove unattached cells, and 100 μl of fresh

RPMI 1640 was subsequently added. Mature biofilm production was documented by staining the polysaccharide structure of the extracellular matrix of biofilms with 0.1% safranin for 5 min and measuring the optical absorbance at 492 nm with a microplate reader (ChroMate 4300; Awareness Technology, Inc., Palm City, FL) (31). The documented biofilm-producing strain *C. albicans* M61, obtained from an infected intravascular catheter and previously extensively studied (32), was used as a control of biofilm formation.

Antifungal agents. The three echinocandins were obtained from their manufacturers: anidulafungin from Pfizer Inc. (New York, NY), caspofungin from Merck and Co. Inc. (Whitehouse Station, NJ), and micafungin from Astellas Pharma Inc. (Tokyo, Japan). Each antifungal was dissolved in sterile water to a final concentration of 3.3 mg/ml for ANID, 5 mg/ml for CAS, and 10 mg/ml for MFG and maintained as a stock solution at −35°C for up to 1 month. A portion from each antifungal stock was further diluted to 1,024 mg/liter in RPMI 1640 and used to prepare a series of 2-fold dilutions, as follows: ANID, 0.007 to 256 mg/liter; CAS, 0.03 to 256 mg/liter; MFG, 0.06 to 256 mg/liter.

Antifungal treatment and assessment of biofilm damage. One hundred microliters of the above-indicated serially double-strength 2-fold-diluted concentrations of each drug was added to corresponding microtiter wells containing 100 μl mature biofilms or planktonic cells, and the plates were incubated at 37°C on a rocker table for an additional 24 h. For controls, biofilms or planktonic cells were incubated in the presence of 200 μl of RPMI 1640 without antifungals under otherwise identical conditions. The *in vitro* activity of each drug against biofilms or planktonic cells was assessed by using an XTT [2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide] reduction assay as described previously (33) with minor modifications. Briefly, 150 μl of XTT (0.25 g/liter; Sigma-Aldrich) containing coenzyme Q (40 mg/liter; Sigma-Aldrich) was added to microtiter plates after a washing step with PBS to remove antifungal agents or growth medium. The plates were then incubated for 30 to 50 min at 37°C, and the change in color, indicating the percent fungal damage effected by each antifungal, was measured in a microtiter plate reader at 450 nm with a reference wavelength of 690 nm. Antifungal activity was calculated according to the following formula: percent fungal damage = $[1 − (X/C)] \times 100$, where X is the absorbance of experimental wells and C is the absorbance of control wells. The MICs for biofilms and planktonic cells were determined as the minimum antifungal concentration that caused ≥50% fungal damage compared to that for the untreated controls (17, 34).

Statistical analysis. Each concentration of ANID, CAS, and MFG for every clinical isolate was tested in pentaplicate, and each untreated control was tested in 16 replicates per experiment. The average values of these replicates were used in the data analysis to determine the mean ± standard error (SE) for each condition. All isolates were retested at least twice in independent experiments. The documented biofilm-producing strain *C. albicans* M61, used as a control, was retested five times on separate days. The differences in biofilm formation between M61 and each *Candida*
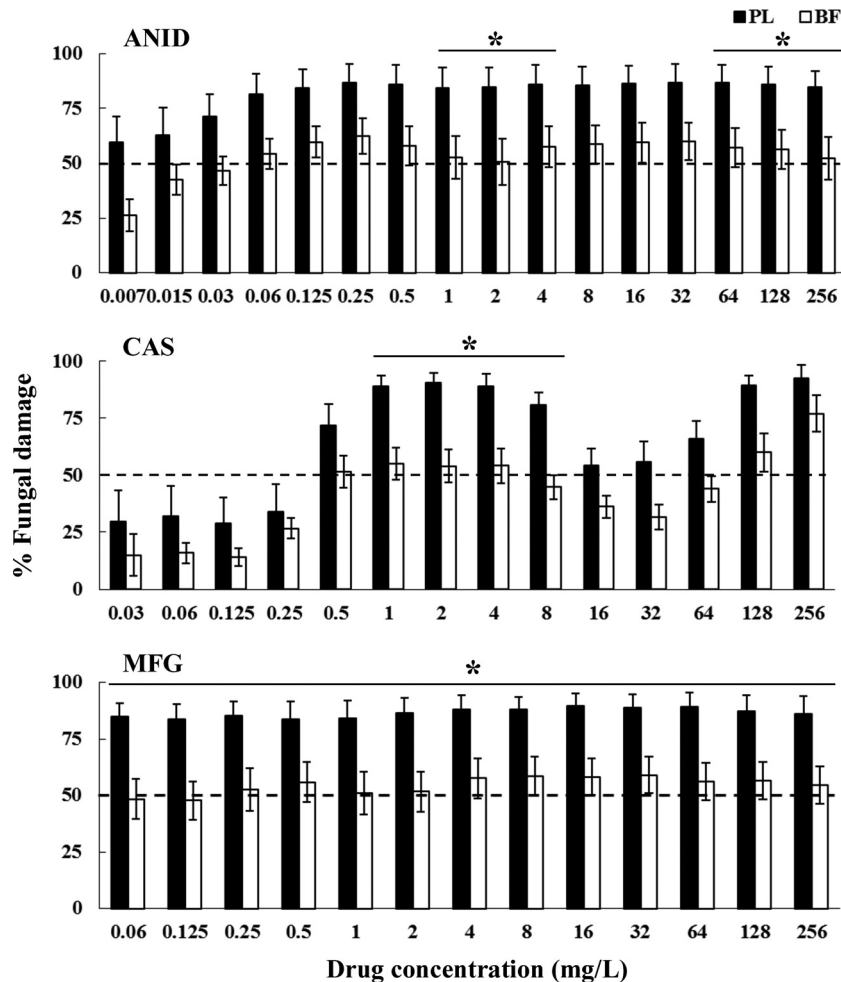
FIG 1 Fungal damage of planktonic cells (PL) and biofilms (BF) of *C. albicans* bloodstream isolates caused by different concentrations of ANID, CAS, and MFG. Fungal damage was assessed by XTT assay. Results are means ± SEs of the percent fungal damage of planktonic cells and biofilms of all isolates of a particular species and drug. Each of the 15 isolates was studied at least twice in independent experiments. Asterisks show significant differences between planktonic cells and the corresponding biofilms for the concentrations indicated by horizontal lines ($P < 0.01$). The discontinuous line denotes the MIC that caused ≥50% fungal damage compared to the untreated controls.

species were examined by the Wilcoxon rank-sum test. The differences in fungal damage between planktonic cells and biofilms of the strains within each *Candida* species caused by ANID, CAS, and MFG and the differences in drug efficacy (MICs) for each echinocandin between planktonic cells and biofilms were assessed by the Wilcoxon matched-pairs test. Data were analyzed using Instat (version 3) biostatistics software (GraphPad Inc., San Diego CA). A two-tailed *P* value of <0.05 was considered significant.

RESULTS

Biofilm formation among *Candida* species. All isolates used in this study were biofilm producers, but the degree of biofilm formation depended on the *Candida* species. In comparison to the biofilm production of the *C. albicans* M61 control strain, as measured by the mean optical density ± standard error of the mean (0.96 ± 0.02), *C. albicans* (0.95 ± 0.01), *C. lusitaniae* (0.94 ± 0.01), and *C. guilliermondii* (0.92 ± 0.02) isolates formed comparable biofilms. In contrast, biofilms of intermediate density compared to the density of the biofilms produced by the control strain were formed by *C. parapsilosis* (0.84 ± 0.02) and *C. krusei* (0.53 ± 0.01) (*P* < 0.01; data not shown).

Susceptibility of *Candida* planktonic cells and biofilms to echinocandins. (i) Planktonic cells. The ANID and MFG MICs for *C. albicans* were ≤0.007 mg/liter and ≤0.06 mg/liter, respectively, whereas the MICs of these antifungals for both *C. krusei* and *C. lusitaniae* were 0.06 to 0.125 mg/liter and ≤0.06 mg/liter, respectively. The patterns of *C. albicans*, *C. krusei*, and *C. lusitaniae* planktonic cell susceptibility to ANID and MFG were similar, with MICs ranging from ≤0.007 to 0.125 mg/liter (Table 1). By comparison, the ANID and MFG MICs for *C. parapsilosis* were 0.03 to 0.125 mg/liter and 0.06 to 0.5 mg/liter, respectively, whereas for *C. guilliermondii* the MICs of these antifungals were 0.5 to 1 mg/liter and 0.06 to 0.125 mg/liter, respectively. Therefore, the corresponding mean MICs of ANID and MFG for *C. parapsilosis* and *C. guilliermondii* were somewhat higher and ranged from 0.06 to 1 mg/liter (Table 1). The MICs of CAS for planktonic cells of *C. albicans* and non-*albicans Candida* isolates were within similar ranges (Table 1; 0.25 to 0.5 mg/liter for *C. albicans*, 0.25 to 1 mg/liter for *C. parapsilosis*, 0.5 to 1 mg/liter for *C. krusei* and *C. guilliermondii*, and 0.25 to 1 mg/liter for *C. lusitaniae*).

The three echinocandins demonstrated similar antifungal ac-
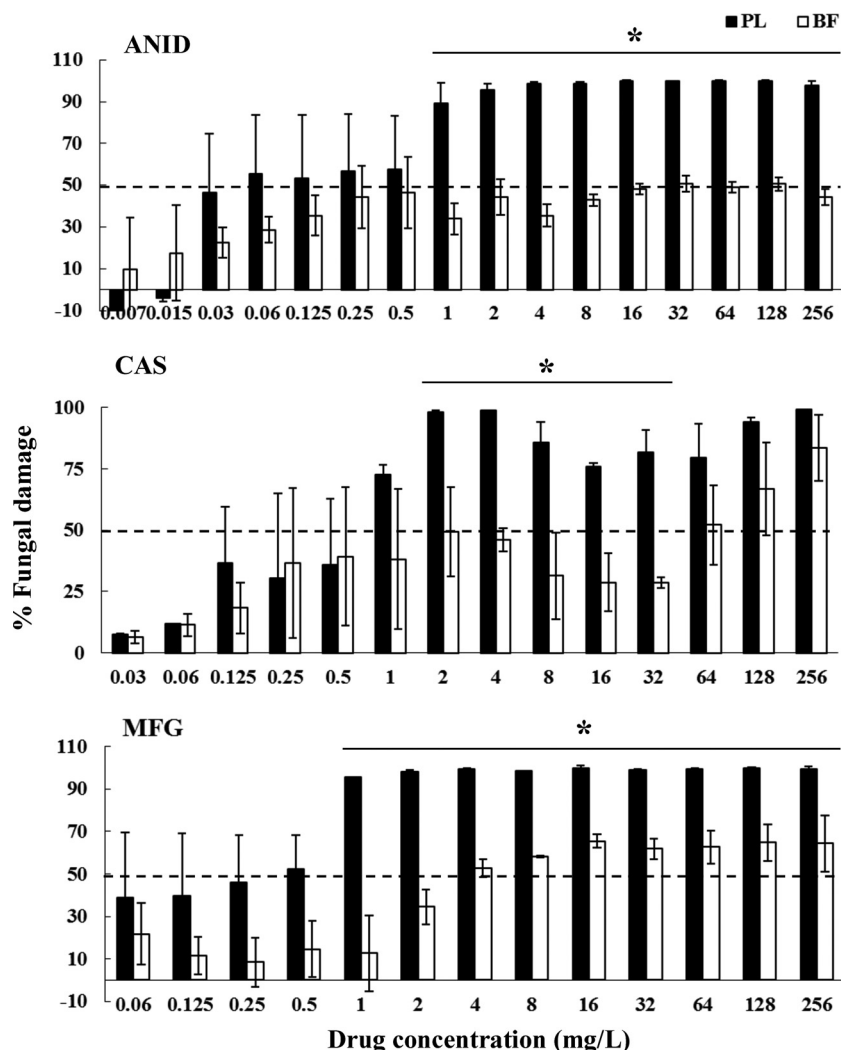
FIG 2 Fungal damage of planktonic cells and biofilms of *C. parapsilosis* bloodstream isolates caused by different concentrations of ANID, CAS, and MFG. Fungal damage was assessed by the XTT assay. Results are means ± SEs of the percent fungal damage of planktonic cells and biofilms of all isolates of a particular species and drug. Each of the 6 isolates was studied at least twice in independent experiments. Asterisks show significant differences between planktonic cells and the corresponding biofilms for the concentrations indicated by horizontal lines ($P < 0.01$). The discontinuous line denotes the MIC that caused ≥50% fungal damage compared to the untreated controls.

tivities against planktonic cells of the 15 *C. albicans* isolates and those of the control M61 strain: ANID MIC, <0.007 versus 0.015 mg/liter; CAS MIC, 0.5 versus 0.25 mg/liter; and MFG MIC, ≤0.06 versus 0.06 mg/liter, respectively, while ANID and CAS exhibited >70% fungal damage against planktonic forms of all *Candida* isolates at ≥1 mg/liter (Fig. 1 to 5), MFG showed high antifungal efficacy at concentrations starting from ≥0.125 mg/liter against planktonic cells of *C. albicans* (Fig. 1; 84% ± 7%), *C. krusei* (Fig. 2; 96% ± 0.8%), *C. lusitaniae* (Fig. 4; 95% ± 1.8%), and *C. guilliermondii* (Fig. 5; 80% ± 5.3%). These results suggested that planktonically grown cells of *Candida* isolates share similar profiles of susceptibility to echinocandins.

(ii) Biofilms. Mature biofilms were more recalcitrant to the three echinocandins than their corresponding planktonic cells, especially those formed by *C. parapsilosis*, *C. lusitaniae*, and *C. guilliermondii* (Table 1; $P < 0.01$). All three echinocandins were active against *C. albicans* biofilms, with MICs of ≤0.5 mg/liter

(Table 1). Significant echinocandin-specific differences in the antifungal activities of ANID, CAS, and MFG were observed against biofilms of isolates of the non-*albicans Candida* spp. While all three echinocandins were characterized by high MICs for *C. parapsilosis* (4 to 64 mg/liter), MFG had the lowest value among them (Table 1; 4 mg/liter; $P < 0.01$). The three echinocandins showed diminished activity against biofilms of *C. krusei*, with the lowest MIC characterizing ANID and MFG (Table 1; 0.125 mg/liter; $P < 0.01$). In contrast, increased activity of the three echinocandins was demonstrated against biofilms of *C. lusitaniae* and *C. guilliermondii*, with MICs of ≥32 to >256 mg/liter (Table 1).

In general, among *Candida* species, *C. albicans* and *C. krusei* biofilms exhibited the lowest MICs of all echinocandins. In contrast, biofilms of *C. parapsilosis*, *C. lusitaniae*, and *C. guilliermondii* were more refractory to the three antifungal agents (Table 1).

A paradoxical growth effect was observed with CAS but not with ANID and MFG, with concentrations ranging from 8 to 64
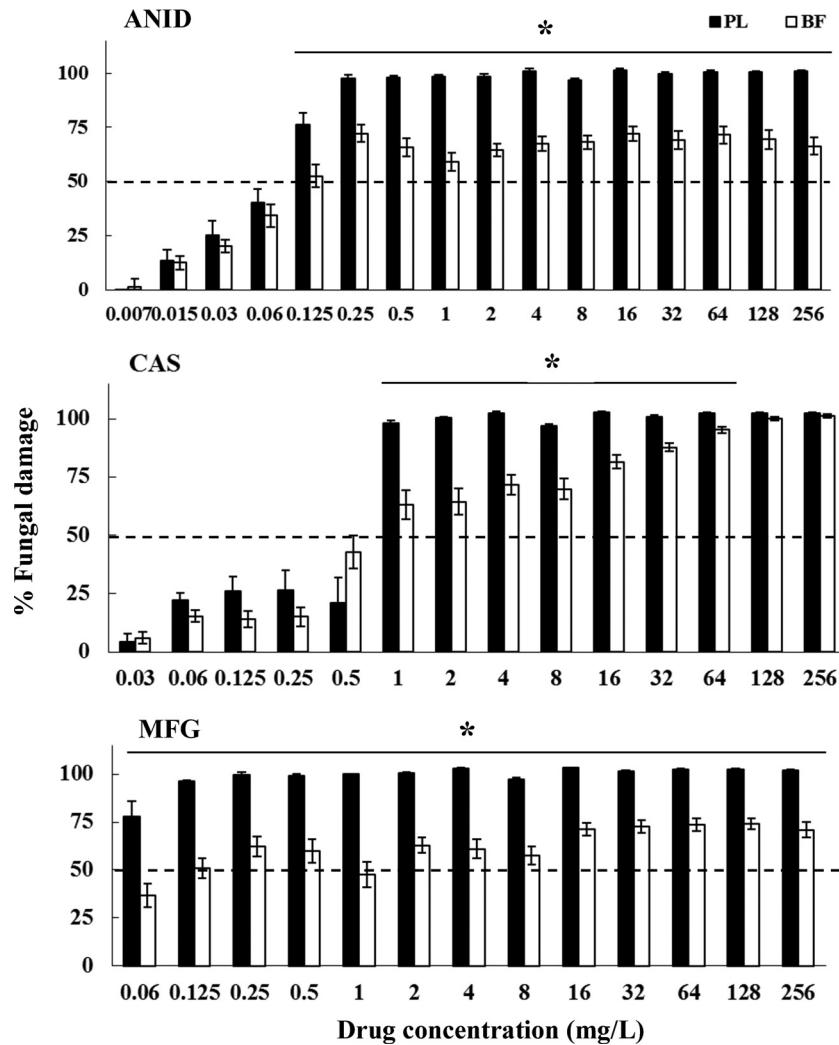
FIG 3 Fungal damage of planktonic cells and biofilms of *C. krusei* bloodstream isolates caused by different concentrations of ANID, CAS, and MFG. Fungal damage was assessed by the XTT assay. Results are means ± SEs of the percent fungal damage of planktonic cells and biofilms of all isolates of a particular species and drug. Each of 12 isolates was studied at least twice in independent experiments. Asterisks show significant differences between planktonic cells and the corresponding biofilms for the concentrations indicated by horizontal lines ($P < 0.01$). The discontinuous line denotes the MIC that caused ≥50% fungal damage compared to the untreated controls.

mg/liter against both planktonic cells and biofilms of *C. albicans* and *C. parapsilosis* but not against *C. krusei*, *C. lusitaniae*, or *C. guilliermondii* (Fig. 1 to 5). Of note, the fungal damage resulting from CAS against biofilms of the last three species was increased with increasing concentrations of CAS (for *C. krusei*, 43% to 100% over the range of 0.5 to 256 mg/liter [Fig. 3]; for *C. lusitaniae*, 29% to 96% over the range of 16 to 256 mg/liter [Fig. 4]; for *C. guilliermondii*, 21% to 81% over the range of 16 to 256 mg/liter [Fig. 5]). Nevertheless, except for the antibiofilm activity of CAS at ≥64 mg/liter for *C. krusei* and at ≥128 mg/liter for *C. lusitaniae*, greater than 90% fungal damage was not observed to be caused by CAS or the other echinocandins for any of the other organisms of this study.

DISCUSSION

Due to their recalcitrant nature toward many antifungal agents and host immune mechanisms (35–37), fungal biofilm-associated

infections have been linked to increased morbidity and mortality rates. *C. albicans* as well as non-*albicans Candida* species have been shown to form biofilms and contribute to the pathogenesis of *Candida* infections (38–40). The activities of individual echinocandins against *Candida* biofilms have been studied. However, in this study, all three echinocandins were compared for their activities against *Candida* biofilms caused by *C. albicans* and non-*albicans Candida* species.

We determined the MIC profiles of ANID, CAS, and MFG for planktonic cells and mature biofilms of *C. albicans*, *C. parapsilosis*, *C. krusei*, *C. lusitaniae*, and *C. guilliermondii*. Comparative analyses demonstrated the presence of species-specific and drug-specific differences in the MICs of the three echinocandins for biofilms. We found that while planktonic cells of *Candida* species were susceptible to the three echinocandins with mean MICs of ≤1 mg/liter, there were differences in the MICs of echinocandins for biofilms among *Candida* species. For the first time, to our
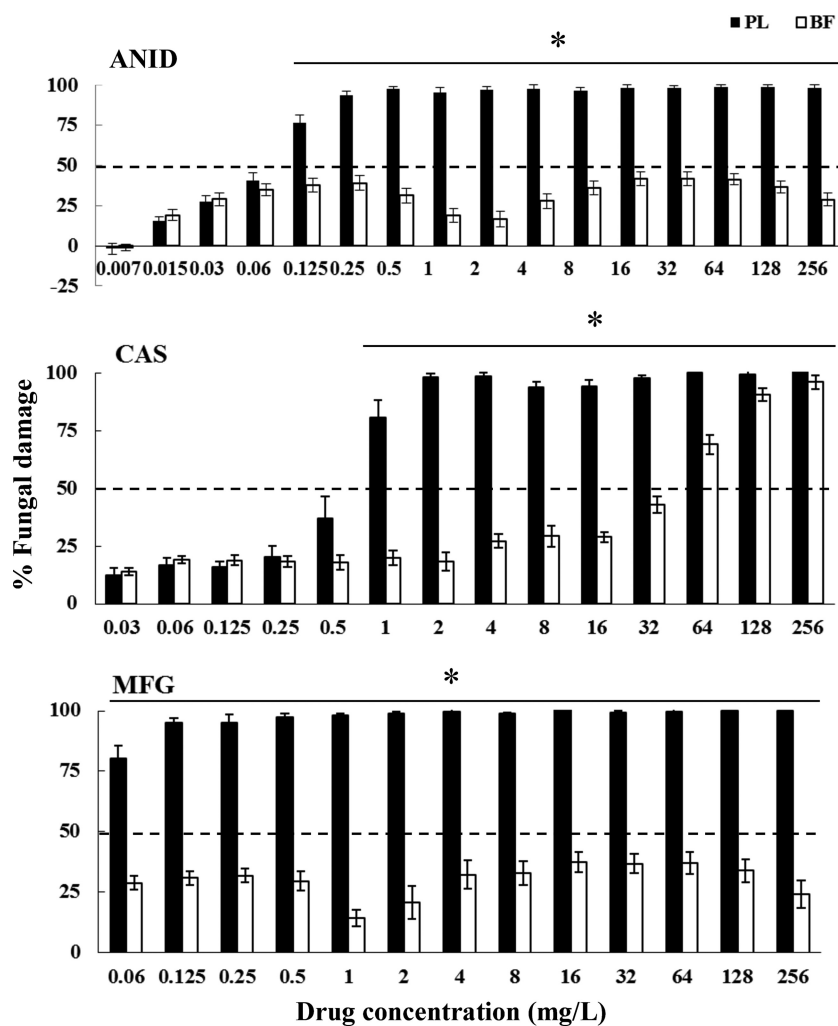
FIG 4 Fungal damage of planktonic cells and biofilms of *C. lusitaniae* bloodstream isolates caused by different concentrations of ANID, CAS, and MFG. Fungal damage was assessed by the XTT assay. Results are means ± SEs of the percent fungal damage of planktonic cells and biofilms of all isolates of a particular species and drug. Each of 16 isolates was studied at least twice in independent experiments. Asterisks show significant differences between planktonic cells and the corresponding biofilms for the concentrations indicated by horizontal lines ($P < 0.01$). The discontinuous line denotes the MIC that caused ≥50% fungal damage compared to the untreated controls.

knowledge, *C. lusitaniae* and *C. guilliermondii* biofilms were reported to have high MICs (≥32 mg/liter) to all three echinocandins. By comparison, *C. albicans* and *C. krusei* biofilms had relatively low MICs (≤1 mg/liter) to the three echinocandins. The *C. parapsilosis* biofilm was generally recalcitrant to echinocandins, but MFG exhibited the lowest MIC among them (4 mg/liter). In contrast, the MICs of CAS were either equal to or up to 6 dilution steps higher than those exhibited by ANID and MFG for planktonic cells of *C. albicans* and non-*albicans Candida* species. With the exception of CAS for planktonic cells of *C. krusei*, the resistance breakpoint MICs for the organisms examined did not exceed the Clinical and Laboratory Standards Institute (CLSI)-approved resistance breakpoint MICs of ≥1 mg/liter for *C. albicans* and of ≥8 mg/liter for *C. parapsilosis*, *C. lusitaniae*, and *C. guilliermondii* (41). Finally, in this study, a paradoxical growth of both planktonic cells and biofilms of *C. albicans* and *C. parapsilosis* was demonstrated with CAS concentrations ranging from 8 to 64 mg/liter; this paradoxical effect of CAS was not observed with *C. krusei*, *C. lusitaniae*, or *C. guilliermondii*.

The *Candida* isolates under investigation are all biofilm producers, but biofilm formation was species dependent, being the highest in *C. lusitaniae*, intermediate in *C. albicans* and *C. guilliermondii*, and the lowest in *C. parapsilosis* and *C. krusei*. These findings are consistent with previous observations (11, 18, 42, 43).

As demonstrated in this study and reported by others, the MIC profiles of *Candida* planktonic cells were within the susceptibility range of echinocandins (16, 18, 21, 44–46). However, in our study, significant differences between planktonic cell MICs and biofilm MICs as well as among biofilm MICs of echinocandins were observed for the *C. albicans* and non-*albicans* species.

The MICs of ANID, CAS, and MFG for *C. parapsilosis* biofilms in our study were 32, 64, and 4 mg/liter, respectively. In the literature, *C. parapsilosis* biofilms have shown variable MIC profiles for the three echinocandins. In a study by Fiori et al. (21), *C. parapsilosis* biofilms exhibited low MICs to ANID, ranging from 0.5 to 2 mg/liter, differing by at least 16-fold from the corresponding MIC value exhibited in our study (32 mg/liter). However, in another study investigating long-term trials of continuous-flow
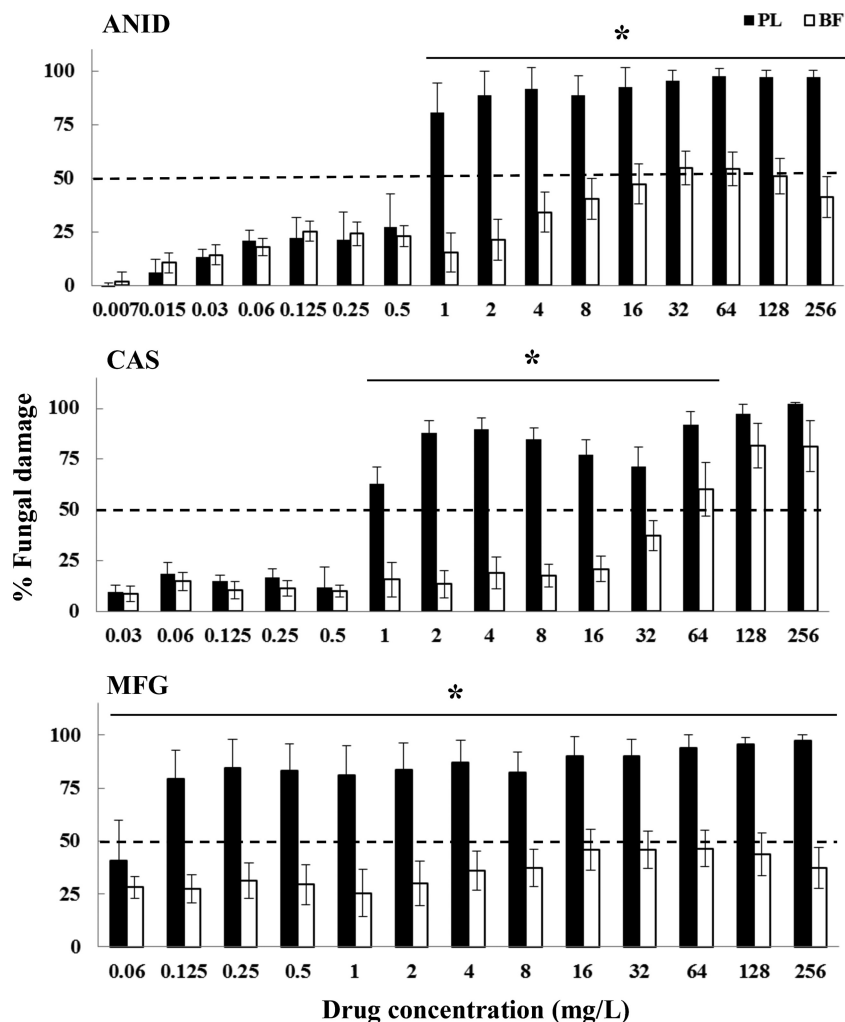
FIG 5 Fungal damage of planktonic cells and biofilms of *C. guilliermondii* bloodstream isolates caused by different concentrations of ANID, CAS, and MFG. Fungal damage was assessed by the XTT assay. Results are means ± SEs of the percent fungal damage of planktonic cells and biofilms of all isolates of a particular species and drug. Each of 5 isolates was studied at least twice in independent experiments. Asterisks show significant differences between planktonic cells and the corresponding biofilms for the concentrations indicated by horizontal lines ($P < 0.01$). The discontinuous line denotes the MIC that caused ≥50% fungal damage compared to the untreated controls.

cultivation, *C. parapsilosis* biofilms demonstrated relatively high MIC profiles (MIC > 32 mg/liter) after 5 days of ANID exposure (47). Likewise, in our study and others, *C. parapsilosis* biofilms exhibited variable CAS and MFG MIC profiles, with MICs ranging from 1 to >16 mg/liter (19, 45, 48, 49). Several explanations for such variability have been offered. Cocuaud et al. have demonstrated that species-specific differences between *C. albicans* and *C. parapsilosis* could depend on the *Candida* species, biofilm age, and concentration of CAS used (50). Seidler et al. (31) have shown that profiles of high MICs could depend on the ability of *Candida* species to form biofilms on different substrates. In particular, the study demonstrated that MFG displays diminished activity (>16 mg/liter) against *C. parapsilosis* mature biofilms grown on polystyrene substrate but increased activity (<0.5 mg/liter) against biofilms grown on central venous catheter sections (31). Finally, interstudy differences could be due to either differences in the biofilm models used or the distinct clumping and irregular appearance of *C. parapsilosis* blastospores embedded within a shal-

low biofilm matrix that results in an easily dissociated biofilm structure (51, 52).

The potential for biofilm formation of *C. lusitaniae* was recently demonstrated by Pannanusorn et al. (53). They observed that *C. tropicalis* and *C. lusitaniae* had the greatest propensity for forming biofilms among all *Candida* spp. studied (53). Recent studies of *C. lusitaniae* indicate that virulence, cell wall formation, and antifungal tolerance are calcineurin dependent (54). Indeed, among echinocandin-resistant isolates, FK506, a calcineurin inhibitor, demonstrated synergistic activity with caspofungin. These observations may carry important implications for treatment and prevention of biofilm formation by *C. lusitaniae*.

Our study demonstrated that *Candida guilliermondii* also has the ability to be highly recalcitrant to the activity of echinocandins in biofilms. Walker and colleagues recently demonstrated that *C. guilliermondii*, *C. krusei*, *C. albicans*, and *C. parapsilosis* upregulate the expression of chitin via calcineurin and protein kinase C in response to subinhibitory concentrations of caspofungin (55).

Such subinhibitory concentrations may be present in biofilms, allowing the increased expression of chitin and increased resistance to the effect of echinocandins. Further contributing to understanding the possible mechanisms of refractoriness of *C. guilliermondii* in biofilms is the study of Barchiesi et al. (56), who found that caspofungin was fungistatic against this organism only for the first 4 to 6 h of the assay, despite being present at a concentration 8 times above the MIC. By the end of the 24-h assay, caspofungin-treated *C. guilliermondii* displayed growth comparable to that of untreated controls. This limited activity contrasted with the more durable inhibitory effect of caspofungin against *C. albicans* and *C. parapsilosis* throughout the 24-h assays (56). The refractoriness of *C. guilliermondii* to echinocandins in biofilms may also be related to this limited activity.

Concerning the paradoxical growth phenomenon observed with CAS in our study and by others (20, 57, 58), the cells have been found to exhibit large amounts of chitin molecules in their cell wall and biofilm matrix, compensating for $(1\rightarrow3)$-β-D-glucan depletion and in this way resisting killing by the antifungal (59, 60). A recent study evaluated the paradoxical growth effect using time-kill experiments and demonstrated that such a phenomenon is eliminated when *Candida* species are briefly exposed to CAS; additional data presented by the same investigators indicate that the paradoxical growth effect is unlikely to have a major impact on the clinical course of patients treated with echinocandins for candidemia (61). However, as suggested by Moriyama et al., the role of the paradoxical effect may be more relevant in the setting of biofilm (60).

In conclusion, our results suggest that echinocandins are active against planktonic cells of all *Candida* species examined and of biofilms of *C. albicans* and *C. krusei* bloodstream isolates. Furthermore, the recalcitrant nature of *C. lusitaniae* and *C. guilliermondii* biofilms has been demonstrated for the first time. Although the two non-*albicans Candida* species were resistant to the antifungal activity of echinocandins, CAS at high concentrations ($\geq$64 mg/liter) was able to damage 70% to 96% of their biofilm structure, therefore being a good candidate for antifungal lock therapy for the containment of fungal growth due to *C. lusitaniae* and *C. guilliermondii* biofilms. The three echinocandins exhibited relatively high MICs for the *C. parapsilosis* biofilm; however, MFG exhibited somewhat greater antifungal activity. Laboratory animal studies are needed to extend these *in vitro* findings to therapeutic interventions for treatment of non-*albicans Candida* species with echinocandins for systemic and antifungal lock therapy. Comparative analysis of the 3-dimensional structure and biofilm-related gene expression between biofilms of *C. albicans* or *C. parapsilosis* and those of *C. lusitaniae* and *C. guilliermondii* may lead to the discovery of new targets for potent antifungal therapy against *Candida* biofilms.

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