Comparative Pharmacodynamics of Echinocandins against *Aspergillus fumigatus* Using an *In Vitro* Pharmacokinetic/Pharmacodynamic Model That Correlates with Clinical Response to Caspofungin Therapy: Is There a Place for Dose Optimization?

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ABSTRACT Echinocandins have been used as primary therapy of invasive aspergillosis (IA), with suboptimal results at standard dosing. Here, we explored the efficacy of dose escalation in a validated *in vitro* pharmacokinetic/pharmacodynamic (PK/PD) model. Six echinocandin wild-type (WT) and three non-WT *A. fumigatus* isolates were tested in an *in vitro* PK/PD model simulating anidulafungin, caspofungin, and micafungin exposures with a free drug maximum concentration (fC_{max}) of 0.01 to 16 mg/liter and a half-life ($t_{1/2}$) of 8 to 22 h. The relationship between the area under the dosing interval time-free drug concentration curve ($fAUC_{0-24}$)/minimum effective concentration (MEC) and % aberrant mycelium formation was analyzed. PK/PD indices associated with 50 to 99.99% maximal activity (EI_{50} to $EI_{99.99}$) were correlated with the clinical outcome of a 50-mg/day standard dose of caspofungin. The probability of target attainment (PTA) was calculated for different dosing regimens of each echinocandin via Monte Carlo analysis. A sigmoidal PK/PD relationship was found for WT isolates with EI_{99} values of 766, 8.8, and 115 $fAUC_{0-24}$/CLSI MEC for anidulafungin, caspofungin, and micafungin, respectively. No aberrant mycelia were observed for non-WT isolates, irrespective of their MEC and drug exposure. The EI_{99}, $EI_{99.9}$, and $EI_{99.99}$ values corresponded to 2-, 3-, and 4-$\log_{10}$ formation of aberrant mycelia and correlated with survival, favorable, and complete response rates to caspofungin primary therapy in patients with IA. A very low PTA ($<13\%$) was found for the standard doses of all echinocandins, whereas a PTA of $\geq90\%$ was found with 100 and 150 mg/day of caspofungin and 1,400 mg/day micafungin against WT isolates. For anidulafungin, the PTA for 1,500 mg/day was 10%. Among the three echinocandins, only caspofungin at 2 or 3 times the licensed dosing was associated with a high PTA. Caspofungin dose escalation might deserve clinical validation.

KEYWORDS antifungal agents, aspergillosis, dose optimization, echinocandin, *in vitro* PK/PD model, pharmacodynamics

Echinocandins are currently used as second-line agents for the treatment of invasive aspergillosis (IA), either alone or in combination therapy (1, 2). Among the three echinocandins, only caspofungin has a licensed indication for the treatment of IA in patients who are refractory to or intolerant of other therapies. Nevertheless, their use as an alternative chemotherapeutic option for the management of increasingly

reported azole-resistant IA is increasing given their excellent tolerability, the rare occurrence of echinocandin resistance among *Aspergillus fumigatus* isolates, and the promising results of a randomized controled clinical trial of azole-echinocandin combination therapy against IA (3). It is of note, however, that caspofungin and micafungin have been used as primary therapy for IA with suboptimal results (~50% favorable response) (4, 5), and breakthrough *Aspergillus* infections emerging during echinocandin treatment have been reported (6–9). These observations call into question the role of echinocandins at standard dosing for the treatment of IA. In a rabbit model of pulmonary aspergillosis, the pharmacokinetic/pharmacodynamic (PK/PD) index associated with the anidulafungin effect using residual fungal burden, lung weight pulmonary infarct score, and survival could not be clearly distinguished (10) whereas the anidulafungin area under the concentration-time curve from 0 to 24 h (AUC_{0-24})/minimum effective concentration (MEC) correlated better with survival in a nonneutropenic murine model of disseminated aspergillosis (11) and caspofungin drug maximum concentration (C_{max})/MEC was the driving PK/PD index in a neutropenic murine model of invasive pulmonary aspergillosis that used PCR to assess pulmonary fungal burden (12).

An integrated understanding of a drug's PK/PD properties is the key of maximizing its therapeutic benefit. However, considering the unique mode of action of echinocandins against *Aspergillus* spp. (formation of aberrant mycelia without complete inhibition of growth), preclinical PD studies for describing exposure-effect relationships and dose optimization are hampered by the lack of a reliable endpoint to quantify antifungal activity. Indeed, animal models have showed conflicting results regarding the driving PK/PD index that best predicts their efficacy (10–13), highlighting the difficulty of *in vivo* PK/PD studies with echinocandins. We have recently described a new *in vitro* endpoint for the activity of echinocandins activity based on the property of *Aspergillus* mycelia to attach on a dialysis membrane (DM) tube after being exposed to echinocandins in an AUC-dependent manner (14). In particular, the proposed marker was easily quantified, reproducible, and amenable for PK/PD studies, while it correlated with *in vivo* survival in a mouse model of *A. fumigatus* infection. Here, we assessed the PD of anidulafungin, caspofungin and micafungin against *A. fumigatus* in an *in vitro* PK/PD model. For each echinocandin, the PK/PD relationship was described and the probability of target attainment (PTA) for different dosing regimens was calculated.

RESULTS

MECs/MICs. The echinocandin MECs/MICs for the included isolates determined by each susceptibility testing methodology are shown in Table 1. All WT isolates had the same CLSI and EUCAST MECs for anidulafungin, caspofungin, and micafungin. For anidulafungin and caspofungin, the XTT [2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide] and MIC test strip (MTS) method revealed 1 and 1 to 2 2-fold-dilution differences in *in vitro* susceptibility, respectively. For micafungin, all isolates had the same XTT and MTS MICs. For non-WT isolates, CLSI and EUCAST MECs ranged from 2 to 16, 4 to 16, and 1 to 8 mg/liter for anidulafungin, caspofungin, and micafungin, whereas for XTT and MTS, the MIC ranges were 2 to 32, 2 to 16, and 0.5 to 8 mg/liter for anidulafungin, caspofungin, and micafungin.

Pharmacokinetics. The simulated free drug maximum concentration (fC_{max}) values for all three echinocandins were within the target values (<20% deviation) with an average (95% confidence internal [CI]) half-life ($t_{1/2}$) of 22.4 h (18.0 to 29.6 h) for anidulafungin, 13.0 h (9.8 to 19.2 h) for caspofungin, and 8.0 h (6.0 11.8 h) for micafungin for all dosing regimens. Representative time-concentration profiles for each echinocandin are depicted in Fig. 1. Anidulafungin, caspofungin, and micafungin simulated dosages resulted in area under the dosing interval time-free drug concentration curve ($fAUC_{0-24}$) values of 1.39 to 278.2, 0.20 to 216.0, and 0.10 to 42.6 mg.h/liter, respectively.

Pharmacodynamics. Regarding the WT isolates, the % of aberrant mycelia attached on DMs was progressively increased at higher drug exposures, with minimal variation among replicates (<9%) (Fig. 2). All non-WT isolates formed regular hyphae like those of the drug-free controls. In particular, no aberrant mycelia were observed

TABLE 1 Echinocandin MECs/MICs determined by different susceptibility testing methods[a] of *A. fumigatus* isolates used in the present study

Antifungal	WT status of isolate	MEC/MIC (mg/liter) by:			
		CLSI	EUCAST	XTT	MTS
Anidulafungin	WT				
	AZN8196	0.016	0.03	0.004	0.004
	V52-35	0.016	0.03	0.008	0.008
	DPLRG101[b]	0.016	0.03	0.004	0.004
	Non-WT				
	DPL1035-homo	4	16	16	2
	DPL32458	2	16	32	8
Caspofungin	WT				
	AZN8196	0.25	0.5	0.25	0.06
	NIH 4215 (ATCC no. MYA-3626)	0.25	0.5	0.5	0.125
	AUH-25	0.25	0.5	0.5	0.25
	AUH-27	0.25	0.5	0.25	0.06
	AUH-29	0.25	0.5	0.25	0.06
	Non-WT				
	DPL1035-homo	16	8	16	8
	DPL32458	16	4	8	2
	DPLRG101[b]	8	4	8	2
Micafungin	WT				
	AZN8196	0.016	0.03	0.008	0.004
	V52-35	0.016	0.03	0.008	0.004
	DPLRG101[b]	0.016	0.03	0.008	0.004
	Non-WT				
	DPL1035-homo	1	4	8	0.5
	DPL32458	8	8	8	1

[a]Broth microdilution (CLSI, EUCAST, and XTT) and gradient concentration strips (MTS).
[b]Demonstrated a non-WT phenotype only to caspofungin.

for non-WT isolates, even at the highest concentrations tested (16 and 4 mg/liter for anidulafungin/caspofungin and micafungin, respectively), irrespective of the different corresponding MECs/MICs. The DPLRG101 demonstrated a non-WT phenotype only to caspofungin, with no formation of aberrant mycelia attached on DMs, in accordance with the elevated MEC compared to those for the other two echinocandins, where aberrant mycelia were observed in accordance with the WT MECs (Fig. 2).

For the WT isolates, near-maximal activity ($\geq$80% aberrant mycelia) was observed at an anidulafungin fC_{max} of $\geq$0.25 and $\geq$0.5 mg/liter for isolates AZN8196/DPLRG101 and V52-35, respectively (Fig. 2). Those isolates had the same CLSI (0.016 mg/liter) and EUCAST (0.03 mg/liter) MECs, but different XTT (0.004/0.004 and 0.008 mg/liter) and MTS (0.004/0.004 and 0.008 mg/liter) MICs, respectively (Table 1). For caspofungin, near-maximal activity was observed at an fC_{max} of $\geq$0.06 and $\geq$0.125 mg/liter for isolates AZN8196/AUH-27/AUH-29 and NIH 4215/AUH-25, respectively (Fig. 2). Despite having identical CLSI (0.25 mg/liter) and EUCAST (0.5 mg/liter) MECs, those isolates exhibited different XTT (0.25/0.25/0.25 and 0.5/0.5 mg/liter) and MTS (0.064/0.064/0.064 and 0.125/0.25 mg/liter) MICs, respectively (Table 1). For micafungin, near-maximal activity was observed at an fC_{max} of $\geq$0.06 mg/liter for all isolates (Fig. 2). Notably, anidulafungin and micafungin effective fC_{max} values are 5 and 2 2-fold dilutions higher than the respective CLSI MECs, while caspofungin fC_{max} values are one to two 2-fold dilutions lower than the CLSI MECs, indicating sub-MEC antifungal effects only for caspofungin. At lower concentrations ($<$MEC of micafungin and anidulafungin and $<$0.25$\times$MEC of caspofungin) healthy hyphae were formed similar to those in drug-free controls and non-WT isolates.

PK/PD relationship. No effect was found even at high echinocandin exposures ($fC_{max} \geq$ 4 mg/liter) against non-WT isolates, independent of their MECs, indicating the absence of traditional exposure-response relationships against non-WT isolates. Therefore, non-WT isolates were excluded from PK/PD analysis. The *in vitro* exposure-
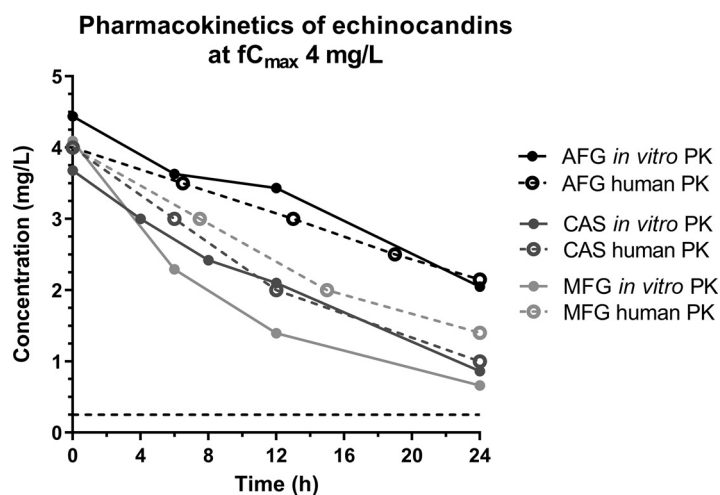
FIG 1 Representative time-concentration profiles for simulated human once-daily dosing regimens of anidulafungin (AFG), caspofungin (CAS), and micafungin (MFG) in an *in vitro* pharmacokinetic/ pharmacodynamic (PK/PD) model with target maximum concentration (fC_{max}) of 4 mg/liter. Data represent drug levels in the internal compartment of the *in vitro* model (solid lines) and the respective target values observed in human serum (broken lines).

effect relationship of the three echinocandins for the WT isolates followed sigmoid curves (R^2 = 0.86 to 0.98; Hill slopes, 2.2 to 4.5) (Fig. 3). The *in vitro* PK/PD relationship of the three echinocandins also followed sigmoid curves (R^2 = 0.85 to 0.97; Hill slopes, 2.2 to 4.5) (Fig. 4). Differences were found among the echinocandins, with caspofungin being the most potent and anidulafungin the least potent. The estimated exposure indices associated with 10 to 99.99% of maximal activity (EI_{10} to $EI_{99.9}$) for CLSI and EUCAST endpoints are presented in Table 2.

Monte Carlo simulation analysis. (i) Clinical correlation. Based on Monte Carlo simulation analysis, the caspofungin cumulative fraction of response (CFRs) for EI_{50}, EI_{80}, EI_{90}, EI_{99}, $EI_{99.9}$, and $EI_{99.99}$ were 99%, 98%, 97%, 89%, 57%, and 31%, respectively, using the *in vitro* CLSI PK/PD targets. The CFRs for $EI_{99.9}$ (57%) and $EI_{99.99}$ (31%) were comparable with the 56% (18/32) favorable (complete plus partial) and 38% (12/32) complete responses, respectively, at the end of treatment of immunocompromised hematological patients with proven/probable cases of pulmonary IA treated with 50 mg of caspofungin as primary therapy in a clinical trial (15). It is of note that the CFR for EI_{99} (89%) correlates with the overall treatment success of 78% (25/32) since the invasive fungal infections (IFI)-related mortality was 22% (8/32) (15).

(ii) Probability of target attainment for standard doses of echinocandins. The proportions of patients attaining the corresponding EI_{99} targets of 766, 8.8, and 115 $fAUC_{0-24}$/CLSI MEC for anidulafungin, caspofungin and micafungin, respectively, are shown in Fig. 5. A low PTA (13%) was found for *A. fumigatus* isolates at the CLSI caspofungin epidemiological cutoff value (ECV) of 0.5 mg/liter (16) for the standard dose of 50 mg, while the PTAs for isolates with micafungin and anidulafungin CLSI MEC_{90} values of 0.016 and 0.03 mg/liter, respectively (17), were 0% with the standard doses of 100 mg.

(iii) Dose optimization. The PTA for *A. fumigatus* isolates at the CLSI caspofungin ECV of 0.5 mg/liter increased significantly when different dosing regimens were explored. In particular, the PTA was 64%, 94%, and 100% for 70, 100, and 150 mg of caspofungin, respectively (Fig. 5). The CFRs based on EI_{99} increased slightly (<10%) with the alternative caspofungin doses (Fig. 6). On the other hand, the CFRs based on $EI_{99.9}$ and $EI_{99.99}$ reached 82%, 92%, and 97% for $EI_{99.9}$ and 49%, 73%, and 90% for $EI_{99.99}$ with the 70-, 100-, and 150-mg doses of caspofungin, respectively (Fig. 6). In contrast, for micafungin, based on EI_{99} a high PTA (90%) for isolates with a CLSI MEC_{90} of 0.016 mg/liter was found only with the highest dose, 1,400 mg, whereas for
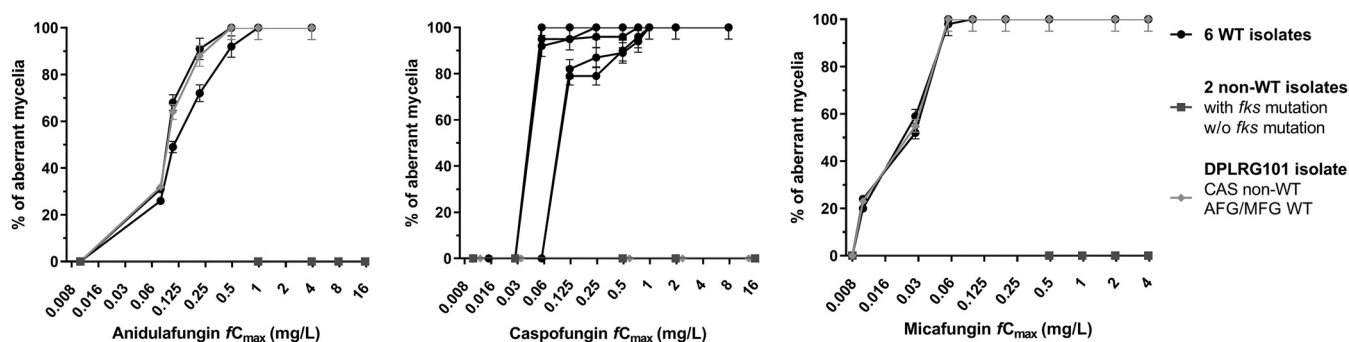
FIG 2 Percentage of *Aspergillus fumigatus* aberrant mycelia attached on dialysis membrane tubes after 48 h of incubation at different echinocandin concentrations. Error bars represent standard deviations (CV <9%). AFG, anidulafungin; CAS, caspofungin; MFG, micafungin.

anidulafungin, the PTA for isolates with a CLSI MEC_{90} of 0.03 mg/liter was just 10% with the highest dose, 1,500 mg (Fig. 5).

DISCUSSION

In the present study, a recently proposed *in vitro* endpoint was used to describe the echinocandins' exposure-response relationships. Although a sigmoidal relationship was found for WT *A. fumigatus* isolates, all non-WT isolates behaved similarly in the *in vitro* model, with no formation of aberrant mycelia irrespective of their MEC and echinocandins' exposure. For the WT strains, the *in vitro* PK/PD targets EI_{99} were 766, 8.8, and 115 $fAUC_{0-24}$/CLSI MEC for anidulafungin, caspofungin and micafungin, respectively. Bridging those PK/PD targets with human PK of standard doses of echinocandins and MEC distribution of WT *A. fumigatus* isolates, a very low PTA (<13%) was found at the ECV and MIC_{90} of all three echinocandins. Simulating higher doses, a PTA of ≥90% was found for WT *A. fumigatus* strains with 100 and 150 mg/day of caspofungin and 1,400 mg/day of micafungin, in contrast to anidulafungin, where a PTA of 10% was found with a dose of 1,500 mg/day.

An interesting observation in the present study was that in addition to supra-MEC exposure-dependent effects observed for all three echinocandins, only caspofungin exhibited a sub-MEC antifungal effect at 0.5 to 0.25×MEC as well. The sub- and supra-MEC effects of caspofungin may be attributed to the dual-uptake model proposed for caspofungin transport into the cell, a high-affinity facilitated-diffusion carrier at low sub-MEC drug levels (≤1 μg/ml) and a nonspecific drug uptake through normal diffusion pathways across the bilayer of the plasma membrane at higher supra-MEC drug levels (18). In agreement with previous concentration-effect relationships (19), among the three echinocandins, caspofungin has the steepest and micafungin and anidulafungin the shallowest exposure-response curves. It is of note that micafungin and anidulafungin have similar structures in their aromatic side chains, whereas caspofungin has an aliphatic side chain. In agreement with our findings, where no aberrant mycelia were found for the non-WT isolates, when microcolonies of a caspofungin non-WT *A.*

TABLE 2 Exposure indices associated with 50 to 99.9% (EI_{50}-$EI_{99.99}$) of maximal activity estimated for each echinocandin and susceptibility testing method based on the E_{max} model

Drug[a]	Method[b]	Mean (95% confidence interval) PK/PD index ($fAUC_{0-24}$/MEC) associated with:					
		EI_{10}	EI_{50}	EI_{90}	EI_{99}	$EI_{99.9}$	$EI_{99.99}$
AFG	CLSI	47 (37–60)	116 (109–125)	287 (232–354)	766 (494–1,188)	1,977 (1,020–3,832)	5,083 (2,028–12,317)
	EUCAST	24 (19–30)	58 (54–62)	143 (116–177)	383 (247–594)	988 (510–1,916)	2,541 (1,049–6,158)
CAS	CLSI	1.9 (1.3–2.8)	3.2 (2.8–3.5)	5.2 (3.6–7.4)	8.8 (4.3–18.2)	14.8 (5.1–43.6)	24.8 (5.9–104)
	EUCAST	1.0 (0.7–1.4)	1.6 (1.4–1.8)	2.6 (1.8–3.7)	4.4 (2.2–9.1)	7.4 (2.5–21.8)	12.4 (3.0–52.2)
MFG	CLSI	6.0 (4.5–8.4)	15.8 (13.9–18.0)	40.8 (33.2–50.2)	115 (74.1–179)	312 (158–616)	843 (336–2,120)
	EUCAST	3.0 (2.4–4.0)	8.2 (7.4–9.2)	22.2 (18.4–26.7)	65.3 (44.3–96.3)	185 (102–335)	521 (234–1,162)

[a]AFG, anidulafungin; CAS, caspofungin; MFG, micafungin.
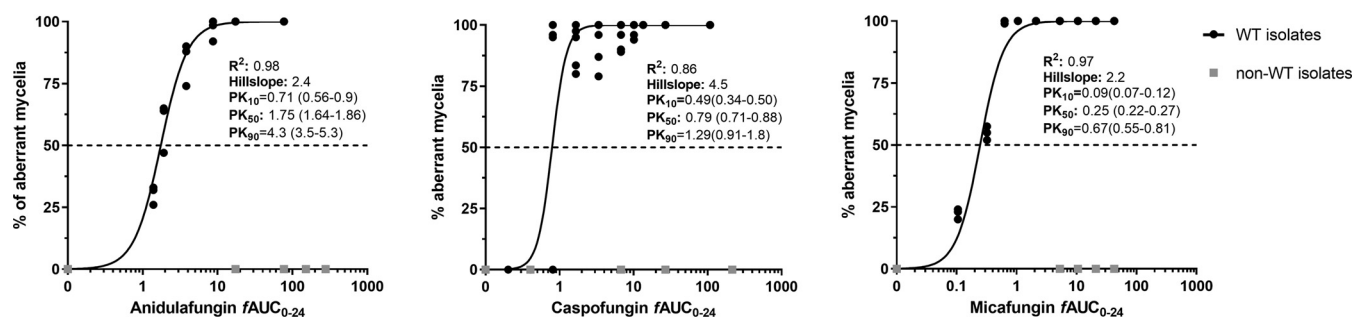[b]Broth microdilution.

FIG 3 *In vitro* exposure-effect relationships of the three echinocandins against *A. fumigatus* isolates.

fumigatus isolate grown on porous aluminum oxide strips were stained with Syto9/propidium iodide, tip lysis was rarely detected at echinocandin concentrations sufficient to cause lysis in WT strains (20). No paradoxical phenomena were observed in the present study, in agreement with previous observations that such phenomena occur at a C_{max} of >8 mg/liter on solid medium and in static liquid cultures (20–22).

The maximal efficacy (75 to 85% 4-day survival) of caspofungin in a temporarily neutropenic (cyclophosphamide IV 1 day before infection) murine model of pulmonary IA was found at plasma total AUC (*t*AUC) (*f*AUC) of 82.5 (2.5) mg · h/liter plasma, which corresponded to an AUC/MEC of 331 (*f*AUC/MEC of 9.9), in line with the effect in the present study (100%) (Fig. 4) (12). Similar findings were observed in a profoundly neutropenic (<100 granulocytes/μl for 10 days) rat model of pulmonary IA, in which *t*AUC (*f*AUC) values of 91.8 (2.8) and 20.1 (0.6) mg · h/liter were associated with 100% and 60% survival, respectively, as predicted from the exposure-response relationship in the present study (100% and ~30%, respectively) (Fig. 3), indicating that the depth of neutropenia does not affect caspofungin efficacy (23). For micafungin, an AUC_{0-24} of 36.33 mg · h/liter (*f*AUC_{0-24} 0.073 mg · h/liter) was associated with 20% 12-day survival in a model of persistently neutropenic (<100 granulocytes/μl with AraC every other day) rabbits infected intratracheally with *A. fumigatus* (13), whereas in a persistently neutropenic (cyclophosphamide every 3 days and cortisone on day 1) mouse model of pulmonary IA, 3 mg/kg/day (*t*AUC ~ 84; *f*AUC ~ 0.17 mg · h/liter [24]) of micafungin for 3 days resulted in 0% survival compared to that with placebo (25), effects that are close to the predicted effects by the exposure-effect relationship of micafungin in the present study (~10% and ~25%, respectively) (Fig. 3). Even in a nonneutropenic but glucocorticoid-suppressed (1 dose before infection) murine model of pulmonary IA, 0% survival was found with doses up to 10 mg/kg (26) (*t*AUC ~ 260 mg · h/liter; *f*AUC = 0.52 mg · h/liter [24]). However, doses of 5 to 10 mg/kg (*t*AUC ~ 138 to 260 mg · h/liter; *f*AUC = 0.28 to 0.52 mg · h/liter [24]) and as low as 1 mg/kg (*t*AUC ~ 30 mg · h/liter; *f*AUC = 0.06 mg · h/liter [24]) in a temporarily neutropenic murine model of pulmonary IA resulted in 100% survival, indicating a profound effect of immune system and particularly of lung effector cells (e.g., alveolar macrophages and dendritic cells) on micafungin efficacy (27, 28). An anidulafungin *f*AUC_{0-24}/CLSI MEC value of 43.3 was
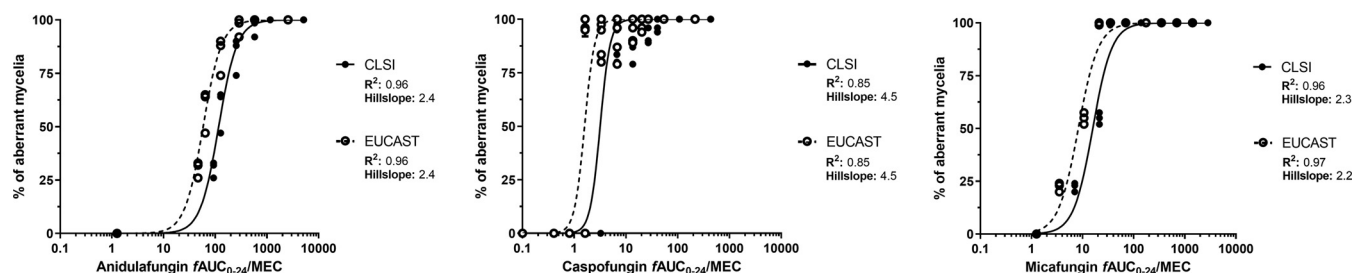

FIG 4 *In vitro* PK/PD relationship of the three echinocandins against wild-type (WT) *A. fumigatus* isolates using the CLSI and EUCAST broth microdilution methodology for assessing the *in vitro* activity of each antifungal. See Table 2 for exposure indices associated with different effects, e.g., 50%, 80%, 90%, etc.
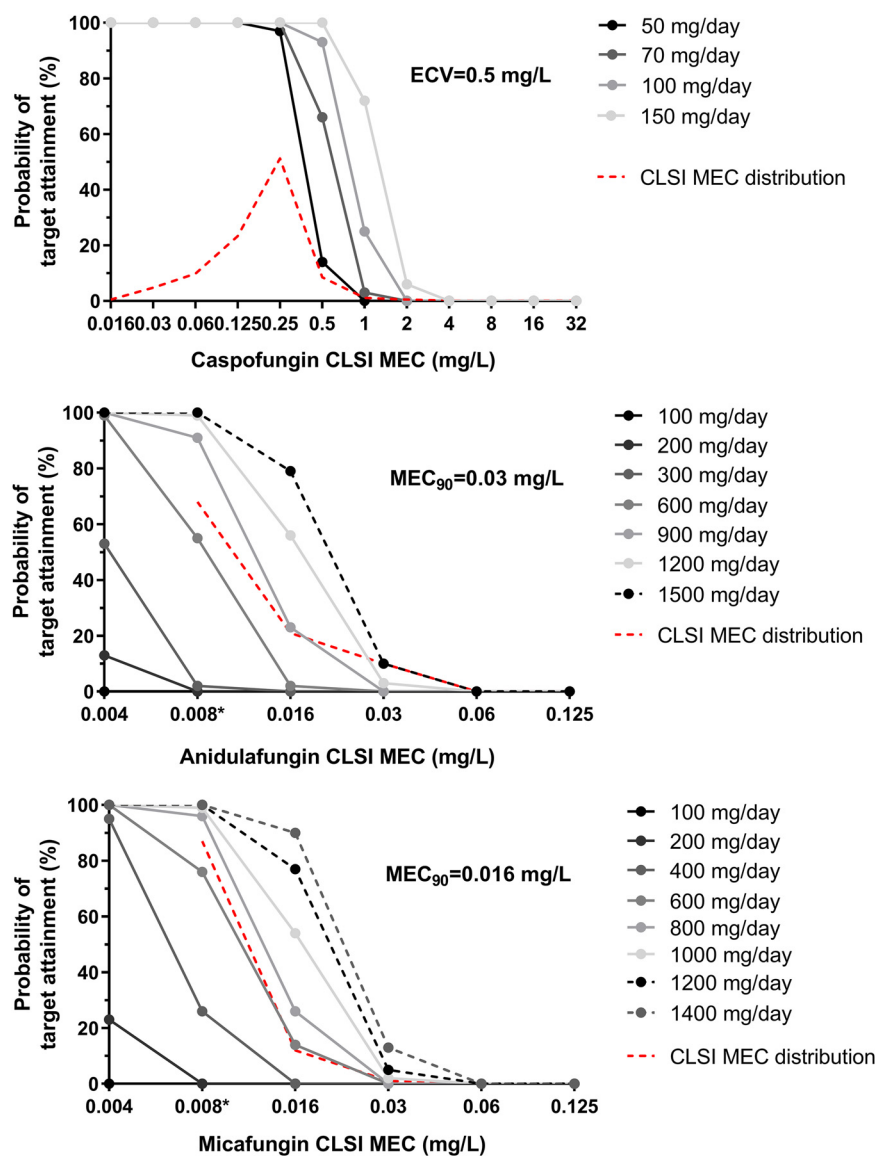
FIG 5 Probabilities of EI$_{99}$ target attainment for 5,000 simulated patients infected with *A. fumigatus* isolates with different CLSI echinocandin MECs and treated with various caspofungin, anidulafungin, and micafungin doses once daily. The CLSI epidemiological cutoff value (ECV) of caspofungin (16) and minimum effective concentration (MEC) values for 90% of isolates tested (MEC$_{90}$) of anidulafungin and micafungin (17) against *A. fumigatus* are shown. The lowest anidulafungin and micafungin concentration tested for the determination of CLSI MEC distribution was 0.008 mg/liter (*) (17).

associated with 18% 23-day survival in transiently neutropenic rats with pulmonary IA (29) and 45% to 73% in a nonneutropenic murine model of disseminated IA with an anidulafungin fAUC$_{0-24}$/EUCAST MEC of 108.7 (11), which are both within the predicted effects based on the exposure-effect relationship in the present study (~10% and ~75%, respectively) (Fig. 4). Thus, the *in vitro* model predicts the efficacy of caspofungin and anidulafungin in animal models of pulmonary IA, which seems not to be affected by underlying immunosuppression, where for micafungin, the *in vitro* model predicts the efficacy only in profoundly neutropenic animal models of pulmonary IA, since micafungin activity was enhanced by the local immune system, as previously found (30).

We have previously shown that the results of the *in vitro* PK/PD model were comparable to the *in vivo* outcome of anidulafungin therapy in a nonneutropenic model of
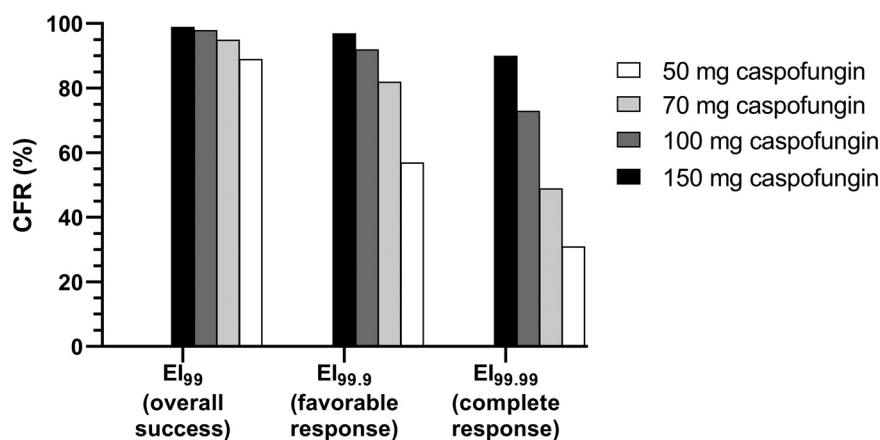
FIG 6 Cumulative fractional responses (CFRs) targeting different PK/PD indices for the standard (50 mg) and alternatives doses of caspofungin. The *in vitro* EI_{99}, $EI_{99.9}$, and $EI_{99.99}$ values correlated with overall success, favorable (complete plus partial), and complete response, respectively, observed in a prospective phase II clinical trial where 50 mg of caspofungin was used as primary therapy for pulmonary invasive aspergillosis (IA) (15).

experimental aspergillosis using the same *A. fumigatus* isolates, as the % of aberrant mycelia attached on DMs corresponded to 14-day survival in mice (14). In the present study, a clinical correlation of the % of aberrant mycelia was found for caspofungin when used as primary therapy against pulmonary IA in immunocompromised patients with hematological malignancies (15). Interestingly, we found that the $fAUC_{0-24}/$ MEC associated with 99% (EI_{99}), 99.9% ($EI_{99.9}$), and 99.99% ($EI_{99.99}$) effects correlated with overall treatment success based on IFI-related mortality, favorable response (complete plus partial, where complete response is the resolution of all clinical signs and symptoms related to IFI with complete radiographic resolution, and partial response is the improvement or resolution of IFI clinical signs and symptom with improvement in radiological abnormalities of >50%), and complete response, respectively. Notably, the EC_{99}, $EC_{99.9}$, and $EC_{99.99}$ values corresponded to 2-, 3-, and 4-log_{10} CFU reductions of unaffected conidia and a simultaneous increase of aberrant mycelia of the initial inoculum (as these two forms of conidia are supplementary, as shown previously [14]), giving a microbiological meaning of those endpoints as well. Since all calculations were made based on free drug levels in blood and because echinocandins are large molecules with small volumes of distribution, the 4-log_{10} effect based on blood levels may result in a 2-, 3-, or 4-log_{10} effect at the site of infection if tissue penetration is 1/100, 1/10, or 1/1, respectively. Furthermore, as PD effects were explored against conidia in the present study, the findings may be more applicable for early treatment, as in the settings of empirical therapy for or prophylaxis from fungal infections in febrile neutropenic patients where caspofungin is indicated.

Despite the fact that the efficacy of caspofungin as a first-line therapy of IA has not yet been evaluated in controlled randomized studies, a literature review paper has identified 7 studies where caspofungin was used as primary therapy against IA (4). Prospective phase II studies where caspofungin standard dose was used as primary therapy for IA reported a median 38% favorable response rate (31, 32), whereas in retrospective analyses and prospective observational registries, a wide range of favorable response rates (27 to 92%; median, 54%) was observed (4) that is similar to the CFR of 57% for the 3-log_{10} CFU reduction of unaffected conidia/aberrant mycelium formation endpoint found in the present study. Of note, voriconazole and liposomal amphotericin B, which are approved as primary therapies against IA, resulted in 53% (21% complete plus 32% partial) (33) and 50% (1% complete plus 49% partial) (34) favorable responses, respectively, which suggests that caspofungin has similar response rates. However, in

both voriconazole and liposomal amphotericin B clinical trials, the 12-week survival rate was 71% (33, 34), which is higher than the 50% to 53% survival rates in the two multicenter prospective phase II studies of caspofungin (standard dose) primary therapy against IA in patients with hematological malignancies or autologous transplants (32) and in allogeneic transplants (31).

Nevertheless, various parameters, such as the underlying disease, neutropenia, and duration of treatment, could explain this difference. Indeed, subset analyses in the AmBiLoad study revealed significant negative associations for the baseline factors of allogeneic stem cell transplantation (12-week survival of 40% versus 71% for no transplantation) and uncontrolled hematological malignancy (12-week survival of 54% versus 81% for controlled malignancy) (34). In a voriconazole trial where the median (range) duration of treatment was 77 days (2 to 84 days), 52% of patients had hematological malignancies and 45% were neutropenic at some point during 2 weeks before the enrollment (33), whereas in the AmBiLoad trial, where the median (range) duration of treatment was 15 days (1 to 60 days), 93% of patients had hematological malignancies (64% uncontrolled) and 71% were neutropenic at baseline (34). In the two multicenter prospective phase II studies of caspofungin (standard dose) primary therapy for IA in patients with hematological malignancies or autologous transplants, the median duration of therapy was 27 days, 85% of patients were neutropenic at start of treatment, 75% had cancer not in remission, and 50% had poor clinical condition (Karnofsky score, <50) (32). In allogeneic transplants, the median duration of therapy was 24 days, and 50% of patients were neutropenic at baseline (31). It is of note that only patients with documented mycological criteria were considered to have IA and were enrolled in the latter studies, as opposed to the AmBiLoad trial (34) and, to a lesser extent, the voriconazole trial (33), in which a high proportion of high-risk patients with halo sign only and without mycological confirmation of IA were enrolled. Thus, in primary therapy studies, caspofungin was given to patients with mycologically confirmed IA for a shorter period than voriconazole (24 days versus 77 days) and in more neutropenic (85% versus 45%) with poor condition patients. Thus, caspofungin may indeed have a role in the management of IA, at least for a subset of patients with similar characteristics as those in the voriconazole clinical trial (33), provided that dose and treatment duration are optimized.

A higher dose of 150 mg/day of caspofungin resulted in an improved favorable response of 86%, which is in line with our finding supporting dose optimization (35). Importantly, no serious adverse events or dose-limiting toxicity were reported in most of the studies assessing caspofungin as first-line therapy for IA, even at higher doses (200 mg/day), classifying it in the category of antifungals with a good safety profile (4). In a dose-escalation study (70 to 200 mg/day of caspofungin) including 44 patients with immunocompromising condition associated with IFI and evidence of IA, the 12-week survival rate was 72% (median duration of therapy, 24.5 days), corroborating our findings (35). However, 28% of late relapses after a 12-week follow-up was observed, highlighting the need for longer treatment with caspofungin (35). Thus, notwithstanding the inevitable variation, the survival and response rates of caspofungin therapy are comparable with those of other antifungal agents licensed as primary therapy for IA. A randomized controlled clinical trial is needed in order to assess the efficacy of higher than the standard dose of 100 and 150 mg of caspofungin against IA.

The efficacy of micafungin monotherapy for the initial treatment of aspergillosis was assessed in a postmarketing survey showing a clinical response of 92/130 (70.8%) patients with micafungin (50 to 300 mg/day), although there was no information on the type of *Aspergillus* infection, and the diagnosis and clinical response were assessed based on the decision of the physician in charge rather than on quantifiable measures (36). A small Japanese multicenter study of 50 to 150 mg/day of micafungin up to 56 days reported an overall clinical response rate (improvements of radiologic invasive shadows and clinical symptoms) of 60% (6/10; 8 with leukemia/lymphoma, only 2 neutropenic) (37) whereas a prospective, open-label, noncomparative, multinational study

with just 12 patients (2 neutropenic at baseline) with IA treated with 121 ± 64.9 mg/day for a mean duration of 61.3 days of micafungin as primary therapy showed no complete response and only 6/12 (50%) patients with partial response, with the remaining patients showing progression (4/12) and stabilization (2/12) (38). Similarly, in a noncomparative, phase IV open-label study with 42 patients with IA based on EORTC-MSG criteria treated with 50 to 300 mg/day of micafungin for up to 12 weeks, no complete and ~50% partial responses were, found with the remaining showing stable disease (~30%) or progression (39). Based on Monte Carlo simulation in the present study, the optimal dose (>90% PTA) of micafungin required to attain the clinically relevant *in vitro* EI_{99} which correlated with overall success is 1,400 mg every 24 h (q24h) for neutropenic patients. Using a stricter endpoint of $EI_{99.9}$, which correlated with favorable response (complete plus partial), a much higher dose of micafungin would be required. Although micafungin has been safely administered in patients undergoing hematopoietic stem cell transplantation at repeated daily doses up to a mean (range) of 600 (442 to 896) mg/day for a median (range) of 18 days (8 to 28 days) with no evidence of dose-related toxicity (40), the safely of such extremely high doses is questionable. Since micafungin PD are greatly improved (>5 to 10 times) in animal models with an active local lung immune response, micafungin may have a role against pulmonary IA in patients recovering from neutropenia or against non-neutropenic patients like those with chronic pulmonary aspergillosis (41). Micafungin may be equally effective as caspofungin as empirical or prophylactic therapy for IFIs, including IA, in neutropenic patients (42, 43) which could be explained by the closer EI_{10} values, i.e., $1-\log_{10}$ effect (if this is the clinically relevant PK/PD target for empirical or prophylactic therapy because fungal load is expected to be low), of the two compounds found in the present study.

Although a *post hoc* analysis of the randomized clinical trial of voriconazole-anidulafungin combination therapy demonstrated that 6-week all-cause mortality was significantly lower in a subset of patients with maximum galactomannan positivity and radiographic findings (-11.5% difference in favor of combination; $P = 0.037$) (44), data regarding anidulafungin monotherapy of IA are lacking. Monte Carlo analysis showed that anidulafungin was not prone to dose optimization, as the PTA was low (10%), even for the highest dose tested (1,500 mg/day).

In conclusion, the previously validated *in vitro* PK/PD model of echinocandins correlated with different clinical outcomes of caspofungin therapy in patients with IA, indicating that the present *in vitro* model's predictions are within the clinical effect ranges. This model can be used to study the PD and optimize exposure of echinocandins against *Aspergillus* spp. This is of great importance given the lack of preclinical models for studying echinocandins and in light of new echinocandin drugs and glucan synthesis inhibitors under development. By no means does the correlation between *in vitro* endpoints and clinical endpoints/outcome indicate that the model can be used to make clinical predictions, but it can be used to generate hypotheses for further clinical investigation. Our results showed that among the three echinocandins, only caspofungin at higher than the standard doses of 100 and 150 mg/day may have a role in primary therapy against IA in neutropenic patients. This hypothesis warrants further clinical verification given the emergence of azole-resistance in *A. fumigatus*.

MATERIALS AND METHODS

Fungal isolates and antifungal susceptibility. A total of six WT molecularly identified *A. fumigatus* clinical isolates with identical CLSI (45) anidulafungin, caspofungin, and micafungin MECs of 0.016, 0.25, and 0.016 mg/liter, respectively, were studied. In addition, the following three non-WT *A. fumigatus* isolates from D. Perlin's laboratory (DPL) collection possessing elevated echinocandin MEC values were tested: the genetically engineered DPL1035-homo (46) isolate with S678P *fks* alteration and CLSI anidulafungin, caspofungin, and micafungin MECs of 4, 16, and 1 mg/liter; the clinical isolate DPL32458 (47) without known *fks* mutation and with CLSI anidulafungin, caspofungin, and micafungin MECs of 2, 16, and 8 mg/liter; and the spontaneous mutant DPLRG101 (47) after exposure to caspofungin without known *fks* alteration and with CLSI anidulafungin, caspofungin, and micafungin MECs of 0.016 (WT), 8 (non-WT), and 0.016 (WT) mg/liter, respectively. Antifungal susceptibility testing was also performed
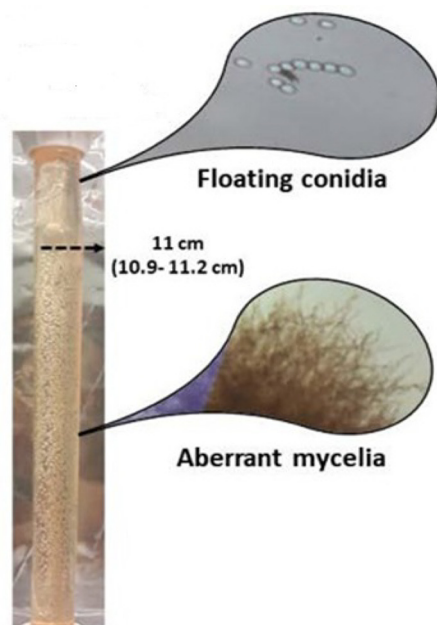
FIG 7 *Aspergillus* conidia after exposure to echincoandins forms, in a dose-dependent manner, aberrant mycelia that are attached to the dialysis membrane tubes (DM). The % of aberrant mycelia can be quantified using the height in the tube (height covered by aberrant mycelia/total height of DM). Unaffected conidia are floating inside the DM and can be quantified with CFU counts (14).

according to the EUCAST recommendations (48) and a recently described XTT [2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide] method based on 24 h 50% inhibition endpoint (49) and using gradient concentration MIC test strips (MTS; Liofilchem, Roseto degli Abruzzi, Italy) based on 24 h complete growth inhibition endpoint according to the manufacturer's instructions. The isolates were stored in normal sterile saline with 10% glycerol at –70°C until use.

In vitro **PK/PD model.** A previously optimized two-compartment dialysis/diffusion closed PK/PD model (14) was used with a 10-ml semipermeable cellulose DM tube with 20-kDa molecular weight cutoff [MWCO] pore sizes (Spectra/Por Float-A-Lyzer G2; Spectrum Laboratories, Inc., Breda, The Netherlands). The DM tube was inoculated with 10^3 CFU/ml conidial suspension in medium (RPMI 1640 [with L-glutamine and without bicarbonate] plus 0.165 M morpholinepropanesulfonic acid [MOPS] supplemented with 100 mg/liter chlorampenicol; AppliChem, Darmstadt, Germany) and placed inside a conical flask with medium that was diluted with fresh medium using a peristaltic pump at a rate equivalent to the drug's elimination rate. The system was incubated on a magnetic heating stirrer at 37°C.

In vitro **pharmacokinetics.** Different anidulafungin, caspofungin, and micafungin exposures with average beta elimination half-lives ($t_{1/2}$) of 26 h (50), 12 h (51), and 15 h (52), respectively, were simulated in the *in vitro* PK/PD model targing a wide range of free drug maximum concentration (fC_{max}) (0.01 to 16 mg/liter of anifulafungin and caspofungin and 0.01 to 4 mg/liter of micafungin) in order to capture both noneffective and highly effective drug exposures. Anidulafungin (Pfizer, Inc., Groton, CT), caspofungin acetate (Merck & Co., Inc., Whitehouse, NJ) or micafungin (Astellas Pharma, Inc., Tokyo, Japan) solution prepared in dimethyl sulfoxide (DMSO; Chem-Lab NV, Zedelgem, Belgium) was injected at the corresponding fC_{max} values in both compartments of the model once daily for 48 h. Drug levels were determined at regular time intervals using microbiological agar diffusion assays for each echinocandin as previously described (53–55). A concentration-time curve was generated for each drug's simulated dose and analyzed by nonlinear regression analysis using the equation $C_t = C_0 \times e^{-kt}$, where C_t (dependent variable) is the concentration of drug at a given time t (independent variable), C_0 is the initial concentration of the drug at $t = 0$ h, e is the physical constant 2.718, and k is the rate of drug removal. The $t_{1/2}$ of each echinocandin was calculated using the equation $t_{1/2} = 0.693/k$ and compared with the respective values observed in human serum. The area under the dosing interval time-free drug concentration curve ($fAUC_{0-24}$) was calculated for each drug's dosing regimen by applying the trapezoidal rule.

In vitro **pharmacodynamics.** The antifungal effect of each echinocandin was determined using the % of aberrant mycelia attached on DMs, which was calculated based on the vertical height (cm) covered by aberrant mycelia (single isolated mycelia were not taken into account) on the DM after 48 h divided by the DM's total height (12.3 cm), as recently described (14) (Fig. 7). We have previously shown that the number of aberrant mycelia affected by echinocandins attached on a DM was inversely related to the number of unaffected conidia floating inside the DM tube, while the 99% effect correlated with a 2-$\log_{10}$ CFU/ml reduction of unaffected floating conidia from the initial inoculum and increased formation of aberrant mycelia (14).
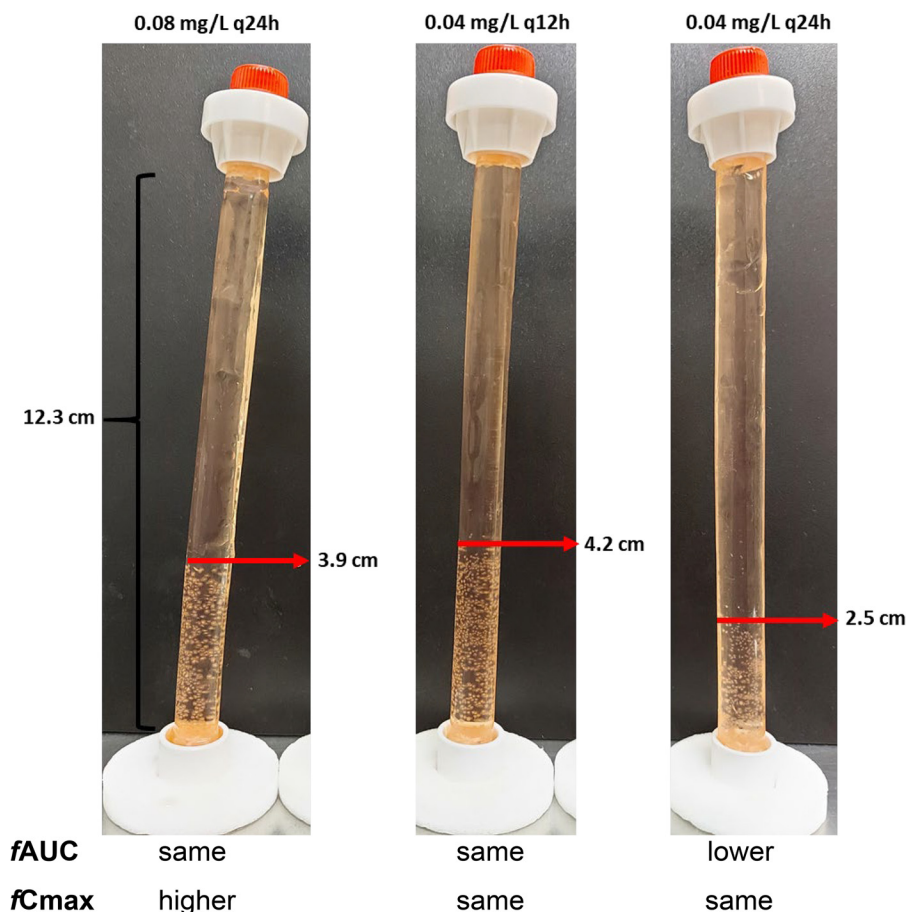
0.08 mg/L q24h **0.04 mg/L q12h** **0.04 mg/L q24h**

12.3 cm

3.9 cm 4.2 cm 2.5 cm

| *f*AUC | same | same | lower |
| *f*Cmax | higher | same | same |

FIG 8 Dose fractionation experiment with suboptimal dosing regimens of anidulafungin (<50% effect) indicates that the $f\mathrm{AUC}_{0-24}$/MEC ratio is the driving PK/PD index. Drug-affected aberrant mycelia are attached on the dialysis membrane in an exposure-dependent manner (the higher the drug exposure, the more of the dialysis tube is covered by those aberrant mycelia). The regimen with 0.08 mg/liter every 24 h (q24h; left photo) exhibited an effect similar to that of a regimen of 0.04 mg/liter q12h (middle photo) (the two regimens had the same AUC but different C_{max} values) and an effect lower than that of the regimen of 0.04 mg/liter q24h (right photo) (the latter regimen had a lower AUC than the other two and the same fC_{max} as the 0.04 q12h regimen).

PK/PD modeling. Previous dose fractionation studies with all three echinocandins showed that their activity is best described by $f\mathrm{AUC}$ rather than by fC_{max} (14). Since in the latter study, highly effective dosing regimens (>50% effect) were used, we conducted further dose fractionation studies using dosing regimens with <50% effect (0.08 mg/liter q24h, 0.04 mg/liter q12h, and 0.04 mg/liter q24h), and the same conclusion was found, i.e., 0.08 mg/liter q24h and 0.04 mg/liter q12h, which had the same AUC but a different C_{max}, were equally and more effective than 0.04 mg/liter q24h (Fig. 8). The *in vitro* relationship of % aberrant mycelia versus $f\mathrm{AUC}_{0-24}$ after 48 h of treatment with each echinocandin was analyzed by nonlinear regression analysis using the sigmoidal E_{max} model with variable slope described by the equation $E = (E_{max} - E_{min}) \times \mathrm{PK}^n/(\mathrm{PK}^n + \mathrm{PK}_{50}{}^n) + E_{min}$, where E_{max} and E_{min} are the maximum (100%) and minimum (0%) effects, respectively, PK is the PK parameter $f\mathrm{AUC}_{0-24}$, PK_{50} is the PK parameter corresponding to 50% of $E_{max} - E_{min}$, and n is the Hill slope. The PK parameters associated with 10% (PK_{10}) and 90% (PK_{90}) of maximal activity was also determined. Furthermore, the *in vitro* exposure-response relationship of each echinocandin was explored by analyzing the *in vitro* relationship of % aberrant mycelia with $f\mathrm{AUC}_{0-24}$/MEC using the E_{max} model as described above. The goodness of fit of the E_{max} model was assessed by visual inspection of graphs, postrun tests, and R^2. Different exposure indices associated with 10%, 50%, 90%, 99%, 99.9%, and 99.99% of maximal activity (EI_{10} to $\mathrm{EI}_{99.99}$) were also estimated for each echinocandin. It is of note that 99%, 99.9%, and 99.99% effect corresponds to a 2-, 3-, and 4-$\log_{10}$ CFU/ml reduction of unaffected floating conidia of initial inoculum and a proportional increase in aberrant mycelia attached on DMs (14).

Monte Carlo simulation. To bridge the *in vitro* data with human PK, Monte Carlo simulation analysis was performed using the normal random number generator function of Excel spreadsheet (Microsoft Office 2010) for patients treated with the standard and alternative intravenous doses of each echinocandin.

(i) Caspofungin. In order to find which is the most clinically relevant EI, the CFRs (expected population probability of target attainment for a certain drug dose and a specific population of microorganisms) were estimated for different EI_{10} to $EI_{99.99}$ values for a previously published CLSI caspofungin MEC distribution of clinical *A. fumigatus* isolates, with a median (range) of 0.25 (0.016 to 32) mg/liter and ECV (MEC_{90}) of 0.5 mg/liter (16), and for the standard caspofungin dose of 50 mg (1) previously used as first-line therapy for the treatment of pulmonary IA in a prospective phase II clinical trial (15). The PTA using the most clinically relevant EI was then calculated for *A. fumigatus* isolates, with CLSI caspofungin MECs of 0.016 to 4 mg/liter (16), using Monte Carlo analysis, for 5,000 hematology patients treated with 50, 70, 100, and 150 mg q24h and attaining steady-state mean $\pm$ standard deviation (SD) AUC_{0-24} values of 117 $\pm$ 28, 175 $\pm$ 56, 250 $\pm$ 80, and 375 $\pm$ 120 mg · h/liter (56, 57), respectively, taking into account the 97% protein binding (51).

(ii) Anidulafungin. The PTA using the most clinically relevant EI was calculated for *A. fumigatus* isolates with CLSI anidulafungin MECs 0.004 to 0.125 mg/liter (17). Monte Carlo analysis was used to simulate 5,000 hematology patients treated with the licensed 100-mg daily dose regimen (1), corresponding to a steady-state mean $\pm$ SD AUC_{0-24} of 110.3 $\pm$ 37 mg · h/liter (58), and with 200, 300, 600, 900, 1,200, and 1,500 mg of anidulafungin q24h, attaining theoretically proportionally increased exposure and the same coefficient of variation with mean $\pm$ SD AUC_{0-24} values of 220.6 $\pm$ 74, 330.9 $\pm$ 111, 661.8 $\pm$ 222, 992.7 $\pm$ 333, 1,323.6 $\pm$ 444, and 1,654.5 $\pm$ 555 mg · h/liter, respectively, considering the 99% protein binding (50) and the linear PK in humans (59).

(iii) Micafungin. The PTA using the most clinically relevant EI was calculated for *A. fumigatus* isolates with CLSI micafungin MECs of 0.004 to 0.125 mg/liter (17). Monte Carlo analysis was performed simulating 5,000 hematological patients treated with the standard 100-mg daily dose regimen (1), corresponding to a steady-state mean $\pm$ SD AUC_{0-24} of 97.1 $\pm$ 29 mg · h/liter (60), as well as with 200, 400, 600, 800, 1,000, 1,200, and 1,400 mg of micafungin q24h, attaining theoretically proportionally increased exposure and the same coefficient of variation with mean $\pm$ SD AUC_{0-24} values of 194.2 $\pm$ 58, 388.4 $\pm$ 116, 663 $\pm$ 212, 776.8 $\pm$ 232, 971 $\pm$ 290, 1,165.2 $\pm$ 348, and 1,359.4 $\pm$ 406 mg · h/liter, respectively, taking into account the 99.8% protein binding (52) and the linear PK in humans (61).

All data were analyzed using the statistics software package Prism version 7.0 for Windows (GraphPad Software, San Diego, CA). All experiments were carried out in duplicate and were independently performed on two different days with individually prepared inocula.

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