Single-Dose Pharmacodynamics of Amphotericin B against *Aspergillus* Species in an *In Vitro* Pharmacokinetic/Pharmacodynamic Model

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Conventional MIC testing of amphotericin B results in narrow MIC ranges challenging the detection of resistant strains. In order to discern amphotericin B pharmacodynamics, the *in vitro* activity of amphotericin B was studied against *Aspergillus* isolates with the same MICs by using a new *in vitro* pharmacokinetic/pharmacodynamic (PK/PD) model that simulates amphotericin B human plasma levels. Clinical isolates of *Aspergillus fumigatus*, *A. terreus*, and *A. flavus* with the same Clinical and Laboratory Standards Institute modal MICs of 1 mg/liter were exposed to amphotericin B concentrations following the plasma concentration-time profile after single-bolus administration with C_{max} values of 0.6, 1.2, 2.4, and 4.8 mg/liter. Fungal growth was monitored for up to 72 h based on galactomannan production. Complete growth inhibition was observed only against *A. fumigatus* with amphotericin B with a C_{max} of ≥ 2.4 mg/liter. At the lower C_{max} values 0.6 and 1.2 mg/liter, significant growth delays of 34 and 52 h were observed, respectively ($P < 0.001$). For *A. flavus*, there was no complete inhibition but a progressive growth delay of 1 to 50 h at an amphotericin B C_{max} of 0.6 to 4.8 mg/liter ($P < 0.001$). For *A. terreus*, the growth delay was modest (up to 8 h) at all C_{max}s ($P < 0.05$). The C_{max} (95% confidence interval) associated with 50% activity for *A. fumigatus* was 0.60 (0.49 to 0.72) mg/liter, which was significantly lower than for *A. flavus* 3.06 (2.46 to 3.80) mg/liter and for *A. terreus* 7.90 (5.20 to 12.29) mg/liter ($P < 0.001$). A differential *in vitro* activity of amphotericin B was found among *Aspergillus* species despite the same MIC in the order *A. fumigatus* > *A. flavus* > *A. terreus* in the *in vitro* PK/PD model, possibly reflecting the different concentration- and time-dependent inhibitory/killing activities amphotericin B exerted against these species.

Amphotericin B (AMB) is an antifungal drug of major importance in the treatment of invasive aspergillosis (1). It is a highly lipophilic and amphoteric molecule that interacts with fungal cell membrane, forming pores and disrupting its integrity (2). Due to its unique mechanism of action, it demonstrates a wide range of pharmacodynamic effects and broad spectrum of antifungal activity. However, conventional MIC testing of amphotericin B resulted in narrow MIC ranges within one to two 2-fold dilutions challenging the detection of resistant strains (3–5). Efforts to develop *in vitro* assays that separate susceptible and resistant strains using richer media or gradient drug concentrations strips were unsuccessful (3, 5). Species-specific epidemiological cutoff values (ECV) have been proposed for amphotericin B and *Aspergillus* spp. based on Clinical and Laboratory Standards Institute (CLSI) broth microdilution methodology, with the *Aspergillus terreus* ECV being one dilution higher than the *A. fumigatus* and *A. flavus* ECV (6).

In addition to inhibitory activity captured by the MIC, amphotericin B exerts a range of different pharmacodynamic effects such as postantifungal effect and concentration-dependent killing (7). All of these effects are usually determined after fungal exposure to constant drug concentrations (2). However, *in vivo*, fungus is exposed to nonconstant amphotericin B concentrations as the drug undergoes metabolism, distribution, and excretion. In particular, its plasma levels follow a triphasic time-concentration profile characterized by the alpha-phase observed within the first 4 h after administration with a half-life of <1 h, the beta-phase observed within 4 to 24 h after administration with a half-life of 6 to 10 h, and the gamma phase observed >24 h of administration with a half-life of >120 h (8). Simulating this time-concentration profile *in vitro* is a challenge because amphotericin B binds to plastic surfaces and degrades over time (9).

We recently developed an *in vitro* model that simulated human pharmacokinetics of antifungal drugs and enabled to study the pharmacodynamics of decreasing drug concentrations as in human plasma (10). This pharmacokinetic/pharmacodynamic (PK/PD) model showed considerable differences of voriconazole activity against *Aspergillus* species which had the same MICs indicating that studying the *in vitro* activity of decreasing drug concentrations provides unique information of pharmacodynamic effects of antifungal drugs (11). With this model, the time- and concentration-dependent pharmacodynamic properties of antifungal drugs can be studied, and PK/PD analysis simulating human pharmacokinetics can be performed.

We therefore studied the activity of amphotericin B against *A. fumigatus*, *A. flavus*, and *A. terreus* strains with similar MICs with the new *in vitro* PK/PD model simulating single-dose pharmacokinetics of amphotericin B in human plasma and monitoring *Aspergillus* growth over time with galactomannan production. Despite the same MICs, important pharmacodynamic differences were found among the three species with amphotericin B being less active against *A. flavus* and *A. terreus* than against *A. fumigatus* reflecting differences in inhibitory, killing, and post-drug exposure effects.

MATERIALS AND METHODS

Strains. Three clinical strains of *A. fumigatus*, *A. flavus*, and *A. terreus* isolated from patients with invasive pulmonary aspergillosis were studied. The MICs, as determined thrice with the CLSI broth microdilution method, were 1 mg/liter for *A. fumigatus*, 1 mg/liter for *A. flavus*, and 1 to 2 mg/liter (mode 1, mg/liter) for *A. terreus* (12, 13). The *A. terreus* strain was included because of its known reduced susceptibility to amphotericin B. The strains were maintained at $-70°C$ in 10% glycerol and cultured twice in Sabouraud dextrose agar at 30°C for 5 to 7 days. A conidial suspension was prepared in normal saline with 1% Tween 20. Conidia were counted with a Newbauer chamber in order to obtain a final suspension 10^5 CFU/ml, and their concentration was confirmed by quantitative cultures on Sabouraud dextrose agar.

Antifungal susceptibility testing. In order to explore the *in vitro* susceptibility of the three isolates by using other methodologies, the isolates were also tested with the gradient concentration strip method Liofilchem MIC test strips (MTS) (Varelas SA, Athens, Greece) according to the manufacturer's instructions and the 2,3-bis {2-methoxy-4-nitro-5-[(sulfenylamino)carbonyl]-2H-tetrazolium-hydroxide} (XTT) methodology as previously described (14). Briefly, for MTS method agar plates with RPMI 1640, morpholinepropanesulfonic acid (MOPS), and 2% glucose were inoculated in three directions with a cotton swab dipped into a 0.5 McFarland conidial inoculum, and the MTS was applied and incubated at 35°C for 24 and 48 h. The MIC was determined as the drug concentration at which the border of the elliptical inhibition zone corresponding to 100% inhibition intersected the strip. For the XTT methodology, 2-fold serial dilutions of amphotericin B in RPMI 1640-MOPS in 96-well flat-bottom microtitration plates were inoculated with 1×10^4 to 5×10^4 CFU/ml, incubated for 48 h when 0.1 mg of XTT/ml and 25 µM menadione was added into each well, and further incubated for 2 h at 35°C when the absorbance at 450 nm was measured. Then, the percent growth in each well was calculated compared to growth in the drug-free control. The MIC was determined as the lowest drug concentration with <10% growth. Furthermore, the minimal fungicidal concentration (MFC) was determined by using an XTT methodology as previously described (13). Briefly, after XTT MIC determination, all clear wells were washed with saline and fresh medium was added. After incubation for 24 h at 35°C, XTT-menadione was added, and the percent growth was calculated based on the absorbance at 450 nm. The MFC was determined as the lowest drug concentration showing <10% growth. All tests were performed three times.

Antifungal drug and medium. Amphotericin B (AMB; Fungizone, Bristol-Myers) was reconstituted at 10,000 mg/liter according to the manufacturer's instructions and stored at $-70°C$. The medium contained 10.4 g of RPMI 1640/liter with glutamine but without sodium bicarbonate (Sigma-Aldrich, St. Louis, MO) and 0.165 M MOPS buffer (pH 7.0; Invitrogen, Carlsbad, CA), with 100 mg of chloramphenicol (Sigma-Aldrich)/liter.

***In vitro* pharmacokinetic/pharmacodynamic model.** The *in vitro* pharmacokinetic simulation model consists of (i) a glass beaker containing 700 ml of medium (external compartment [EC]), in which is placed (ii) a 10-ml dialysis tube (internal compartment [IC]), the wall of which consists of cellulose permeable membrane, allowing the free diffusion of molecules with a molecular mass of <20 kDa, and (iii) a peristaltic pump (Minipuls Evolution, Gilson, France), which removes the content of EC and adds medium at a rate equivalent to drug removal from human serum (10). The conidial suspension is inoculated in the IC within which the growing fungus and its derivative galactomannan (molecular mass, 20 to 60 kDa) remain trapped, whereas nutrients and drug diffuse freely between the IC and the EC. The concentration of the galactomannan increases with fungal growth. The drug is injected into the EC, and its concentration is adjusted by the pump to correspond to the average half-life observed in human plasma after the intravenous administration of amphotericin B. The EC was covered with aluminum foil in order to minimize light exposure and placed on a heated magnetic stirrer (37°C). Before starting each experiment, the temperature and flow rate were controlled. All experiments were repeated twice.

Determination of amphotericin B concentrations. The drug levels in the IC were determined by a microbiological method using the strain *Paecilomyces variotii* ATTC 22319, which is susceptible to AMB (15). Specifically, *P. variotii* conidia at final concentration 5×10^4 CFU/ml were inoculated into prewarmed at 54°C RPMI 1640 medium plus MOPS with 15 g of agar/liter and poured into plastic plates (10 by 10 cm). After solidification of the agar, 1-cm-diameter holes were opened and filled with 100 µl of known drug dilutions (range, 0.25 to 16 mg/liter), as well as 100 µl of IC samples. The plates were incubated at 35°C for 24 h when the diameters of inhibition zones were measured. Unknown drug concentrations in the IC samples were determined using the standard curve constructed from known drug concentrations and corresponding diameters of inhibition zones.

Pharmacokinetic analysis. Several clinically relevant AMB doses were simulated in the *in vitro* model with C_{max} values in human plasma of 0.6, 1.2, 2.4, and 4.8 mg/liter (8, 16, 17). After we took into account any loss of amphotericin B during the experiments due to degradation and surface binding, we adjusted the flow rate in order to approximate the plasma concentration profile of amphotericin B in humans with an alpha phase with a short half-life of <1 h observed within 4 h after drug administration, followed by a beta phase with a longer half-life of 6 to 10 h observed 4 to 24 h after drug administration and a gamma phase with a half-life of >120 h observed >24 h after drug administration (8). Amphotericin B concentrations were determined at 0, 4, 6, 8, 24, 44, 48, and 72 h after the introduction of the drug in the IC using the bioassay. The data were analyzed by nonlinear regression based on a three-compartment model described by the equation $C = C_\alpha e^{k\alpha t} + C_\beta e^{k\beta t} + C_\gamma e^{k\gamma t}$, where k_α, k_β, and k_γ are the rate constants, C_α, C_β, and C_γ are the *y* intercepts for the alpha, beta, and gamma phases, respectively, and C is the concentration at a given time *t*. The half-lives of the alpha, beta, and gamma phases were calculated for EC and IC separately using the equations $t_{1/2,\alpha} = k_\alpha/\ln(2)$, $t_{1/2,\beta} = k_\beta/\ln(2)$, and $t_{1/2,\gamma} = k_\gamma/\ln(2)$, respectively, and were compared to the corresponding values observed in human plasma.

Determination of fungal growth. Fungal growth in the IC was assessed in samples of 100 µl at regular time intervals by determining galactomannan production using an enzyme-linked immunosorbent assay (Platellia; Bio-Rad, Athens, Greece). Samples were diluted with 200 µl of saline in order to reach a final volume of 300 µl before processing. The results were expressed as a galactomannan index (GI) according to the manufacturer's instructions. Galactomannan levels were also determined in the EC in order to ensure that no galactomannan had escaped from the IC.

Real-time PCR conidial equivalent was used as an alternative biomarker of fungal growth and killing. *Aspergillus* DNA was extracted from 200-µl samples from the IC of the *in vitro* PK/PD model after 0 and 72 h with the Qiagen DNA blood minikit (Roche Diagnostics, Athens, Greece) after enzymatic (incubation with proteinase K at 56°C for 10 min) and mechanical (1-min vortexing with glass beads) extraction as previously described (18). Real-time PCR was performed with a previously described assay (2Asp assay) using *Aspergillus* specific primers (ASF1 and ADR1) and probe (ASP28P) (19). The threshold cycle (C_T) of each sample, which identifies the cycle number during PCR when fluorescence exceeds a threshold value determined by the software, was converted to conidial equivalent (CE) *A. fumigatus* DNA by interpolation from a six-point standard curve of C_T values obtained with 10^3 to 10^8 *Aspergillus* CFU/ml. The reduction of the PCR CE after 72 h of incubation compared to 0 h was calculated for each species and amphotericin B dose.

Pharmacodynamic analysis. The *in vitro* pharmacodynamics of each amphotericin B dose and *Aspergillus* species were determined based on the GI-time relationship analyzed with the E_{max} model: $E = E_{min} + E_{max} \cdot T^\gamma/(T^\gamma + T_{50})$, where E is the GI (dependent variable), E_{max} and E_{min}, are the maximum and minimum GIs, respectively, T is the incubation time (independent variable), T_{50} is the time corresponding to 50% of the E_{max},

and γ is the slope of the curve. In addition, the area under the GI-time curve (AUC_{GI}) was calculated for each amphotericin B dose. As shown previously, the parameters E_{max}, γ, and T_{50} describe the extent, rate, and time of galactomannan production, respectively, whereas the AUC_{GI} is a surrogate marker of fungal growth (10, 11). The higher the AUC_{GI}, the greater is the fungal growth. The percentage of fungal growth at each dose was calculated based on the AUC_{GI} of each dose divided by the AUC_{GI} of the growth control. Based on all of these parameters, the *in vitro* activity of amphotericin B dose against each *Aspergillus* species was estimated. Finally, the *in vitro* PK/PD relationship AUC_{GI} versus C_{max} was plotted for each species and analyzed by using the E_{max} model.

Statistical analysis. All analysis was performed with the software Prism 5.01 (GraphPad, Inc., La Jolla, CA). All E_{max} models were globally fitted to the data with E_{max} and E_{min} shared among data sets. Comparisons between E_{max} model parameters of different amphotericin B doses and *Aspergillus* species were assessed using an extra sum-of-squares F test. A P value of <0.05 was considered statistically significant.

RESULTS

Antifungal susceptibility testing. The MTS MICs for *A. fumigatus*, *A. flavus*, and *A. terreus* were 0.75 mg/liter, 2 to 3 mg/liter, and 1 to 1.5 mg/liter after 24 h and 2 to 3 mg/liter, >32 mg/liter, and >32 mg/liter after 48 h, respectively. The XTT MICs and MFCs were 1 to 2 mg/liter and 1 to 2 mg/liter for *A. fumigatus*, 2 to 2 mg/liter and 2 to 4 mg/liter for *A. flavus*, and 1 to 2 mg/liter and 8 to 16 mg/liter for *A. terreus*, respectively.

Bioassay for amphotericin B. The amphotericin B concentrations detected with the bioassay ranged from 0.25 to 16 mg/liter, and the lowest limit of detection was 0.25 mg/liter. Across all experiments performed on the same and different days, the diameter of inhibition zone correlated linearly with the amphotericin B concentration ($r^2 > 0.77$, global $r^2 = 0.84$). The coefficient of variation ranged from 5 to 25% (average, 15%) for all concentrations.

Pharmacokinetic analysis. The C_{max} values in the IC were 0.76 to 0.78, 1.05 to 1.10, 2.5 to 2.7, and 3.9 to 4.4 mg/liter and the AUCs were 4.5 to 5, 8 to 8.6, 31.9 to 33.2, and 64.8 to 67.9 mg · h/liter, respectively, with $t_{1/2,\alpha}$ = 0.2 to 2 h, $t_{1/2,\beta}$ = 10 to 17 h, and $t_{1/2,\gamma}$ = 71 h for the simulated amphotericin B doses with C_{max} values of 0.6, 1.2, 2.4, and 4.8 mg/liter, respectively. Because of the low detection limit of the bioassay, a gamma phase was observed only for the highest dose of amphotericin B with a C_{max} of 4.8 mg/liter. These values were within those observed in human plasma with the largest deviations observed at lower doses (Fig. 1).

Pharmacodynamic (PD) analysis. The GI-time curves were described very well with the E_{max} model ($R^2 > 0.86$), and they were characterized by the same E_{max} but with different slopes and T_{50}s for the different amphotericin B doses and *Aspergillus* species. Among all species and doses tested, complete inhibition of galactomannan production was observed only against *A. fumigatus* with amphotericin B doses corresponding to a C_{max} of ≥2.4 mg/liter (Fig. 2). At lower doses, a significant delay in galactomannan production was observed with mean ± the standard error of the mean T_{50} values of 38.1 ± 2.3 h for C_{max} 0.6 mg/liter and 57.9 ± 2.7 h for C_{max} 1.2 mg/liter compared to 4.2 ± 0.4 h for the drug-free control (P < 0.001). For *A. flavus*, there was no complete inhibition but a progressive delay of galactomannan production with increasing amphotericin B doses since the T_{50} increased from 8.2 ± 0.6 h for the growth control to 9.3 ± 0.6 h at amphotericin B doses with a C_{max} of 0.6 mg/liter, 24.3 ± 3.2 h at a C_{max} of 1.2 mg/liter, 36.7 ± 3.1 h at a C_{max} of 2.4 mg/liter, and 57.8 ± 2.7 h at
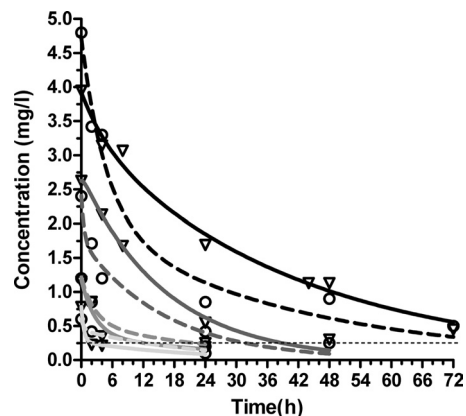

FIG 1 Pharmacokinetic analysis of simulated amphotericin B doses in humans (dashed lines) and in the *in vitro* pharmacokinetic/pharmacodynamic model (solid lines) with C_{max} values of 0.6 mg/liter (light gray), 1.2 mg/liter (medium gray), 2.4 mg/liter (dark gray), and 4.8 mg/liter (black), respectively. Symbols: ▽, IC; ○, expected.

a C_{max} of 4.8 mg/liter (P < 0.001). For *A. terreus*, the delay in galactomannan production was modest since the T_{50} of 4 ± 0.4 h for the growth control increased to 12.6 ± 3.3 h at the highest dose of amphotericin B with a C_{max} of 4.8 mg/liter (P = 0.013). The differences among the tree species in galactomannan production with the two high doses of amphotericin B with C_{max} values of 2.4 and 4.8 mg/liter were confirmed with real-time PCR results with PCR CE at 72 h being reduced by 0.7 and 0.8 log_{10}CE of *A. fumigatus*, 0.1 and 0.4 log_{10}CE of *A. flavus* and increased by 1.5 and 0.1 log_{10}CE of *A. terreus*, respectively (data not shown).

Finally, the *in vitro* activity of amphotericin B against the three *Aspergillus* species was compared by constructing PK/PD curves. In order to quantify the effect of each amphotericin B dose at the entire 72-h period of incubation, the AUC_{GI} was calculated for each dose and species as a surrogate marker of fungal growth and plotted against the corresponding C_{max} values (Fig. 3). The *in vitro* PK/PD relationship followed a sigmoid pattern (global $R^2 = 0.99$). The C_{max} (95% confidence interval) associated with 50% activity for *A. fumigatus* was 0.60 (0.49 to 0.72), which was statistically significant lower than the corresponding C_{max} values against *A. flavus* (3.06 [2.46 to 3.80]) and *A. terreus* (7.90 [5.20 to 12.29]) (P < 0.001).

DISCUSSION

Important pharmacodynamic differences of amphotericin B against the three *Aspergillus* species were found in the recently developed *in vitro* PK/PD model where conidia were exposed to decreasing drug concentrations simulating the plasma concentration-time profile of amphotericin B. Despite the same MICs, the strongest *in vitro* activity of amphotericin B was found against *A. fumigatus*, followed by *A. flavus* and *A. terreus*. The XTT and gradient concentration strip methods showed minor differences in the MICs for the three isolates which clustered within one to two 2-fold dilutions, emphasizing the problem of narrow amphotericin B MIC ranges with these techniques. However, the 48-h MTS MICs were similar for *A. flavus* and *A. terreus* and lower for *A. fumigatus*, whereas XTT MFCs were similar for *A. fumigatus* and *A. flavus* and higher for *A. terreus* advocating for the different pharmacodynamic effects amphotericin B possessed against different *Aspergillus* species.
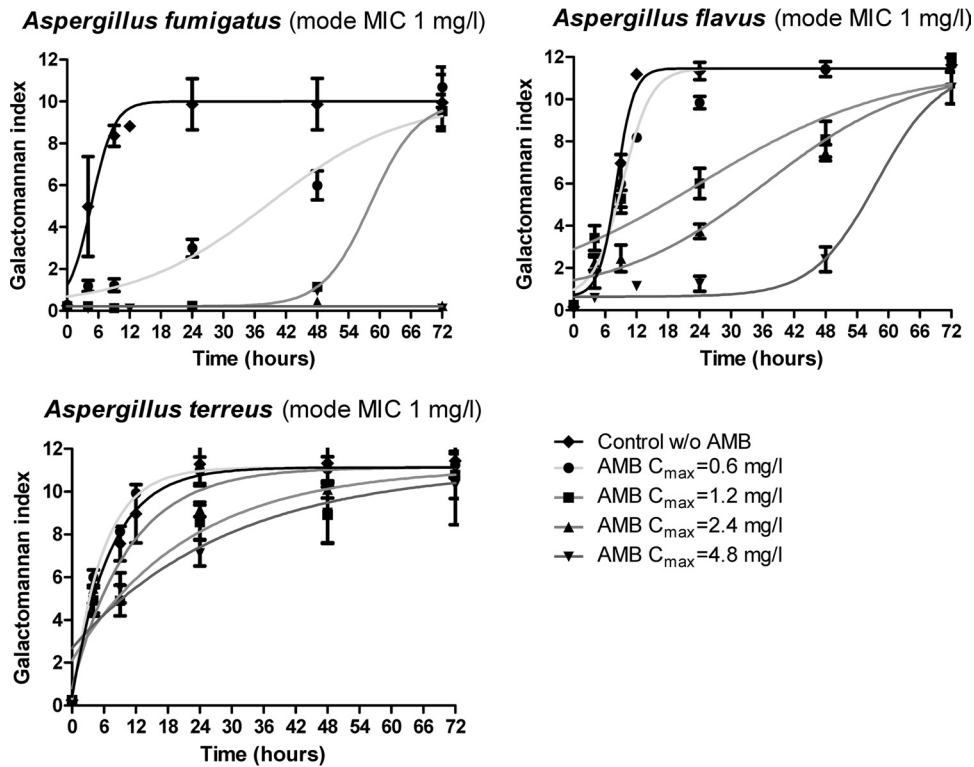
FIG 2 Single-dose pharmacodynamic analysis of simulated amphotericin B doses with C_{max} values of 0.6, 1.2, 2.4, and 4.8 mg/liter against *A. fumigatus*, *A. flavus*, and *A. terreus* isolates with a CLSI mode MIC of 1 mg/liter, as determined by using the GI in the *in vitro* PK/PD model.

Studying the effects of decreasing concentrations of antifungal drugs may provide information about pharmacodynamic properties related to the sub-MIC effect, the postantifungal effect, and time- and concentration-dependent activities. These effects can be quantified by a surrogate marker of fungal growth based on galactomannan production kinetics that captures any difference regarding the antifungal effects described above. Differential antifungal activity was also previously found with the present *in vitro* PK/PD model for voriconazole against the three *Aspergillus* species with identical MICs emphasizing the importance of studying nonconstant drug concentrations (10, 11).

The findings of the present study are in agreement with previous time-kill assays wherein supra-MIC (i.e., 4× and 20× the

FIG 3 Single-dose exposure-efficacy relationship of amphotericin B against each *Aspergillus* species with CLSI mode MICs of 1 mg/liter for *A. fumigatus*, *A. flavus*, and *A. terreus* in the *in vitro* PK/PD model simulating amphotericin B human plasma levels based on the increasing amphotericin B C_{max} values (maximum concentration) and the GI as a marker of fungal growth. Symbols: ●, *A. fumigatus*; ■, *A. flavus*; ▲, *A. terreus*.

MIC) concentrations of amphotericin B killed *A. fumigatus* but not *A. terreus* (20). MFCs of amphotericin B were similar against *A. fumigatus* and *A. flavus* and much higher against *A. terreus* (13). However, MFC/MIC ratios for *A. fumigatus* were 1 twofold dilution lower than those for *A. flavus*, differentiating the *in vitro* activity of amphotericin B against these two species (13). In addition, the three species were previously found to differ also in the post-drug exposure effects since 4× and 1× the MIC of amphotericin B demonstrated a postantifungal effect of >4 h against *A. fumigatus* and of <4 h against *A. flavus* and *A. terreus* (21). The time-dependent activity of amphotericin B inhibition also differed among the three *Aspergillus* species (22). Exposure of *Aspergillus* conidia to supra-MICs for 8 h resulted in a significant amount of metabolic activity for *A. terreus* (16%), less so for *A. flavus* (8%), and even less for *A. fumigatus* (5%). Furthermore, despite the same concentration-effect curves of amphotericin B for *A. fumigatus* and *A. flavus* at 48 h, the inhibitory concentration-effect curve after 8 h of exposure to amphotericin B were shifted to the left for *A. fumigatus* but not for *A. flavus*, indicating that amphotericin B activity is faster against *A. fumigatus* than *A. flavus* species (22).

Taking into account all of these different effects exerted by amphotericin B, the order of amphotericin B *in vitro* activity demonstrated by the present model (*A. fumigatus* > *A. flavus* > *A. terreus*) could be explained by a fast inhibitory action and increased killing rate against *A. fumigatus*, a slower inhibitory action and reduced killing efficiency against *A. flavus*, and the slowest inhibitory action and no killing against *A. terreus*, as was also found with real-time PCR results. In particular, the delayed galac-
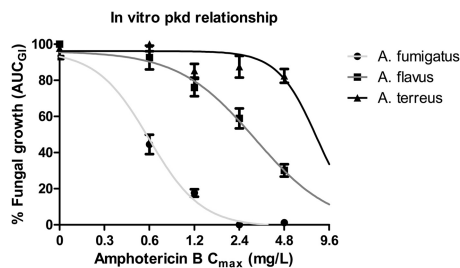
tomannan production of *A. fumigatus* but not *A. flavus* at a lower-than-the-MIC C_{max} of 0.6 mg/liter indicates a strong sub-MIC effect of amphotericin B against the former species. Although there are no data on sub-MIC effects of amphotericin B against *Aspergillus* spp., such effects were described against *Candida* spp. (23). At a C_{max} of 1.2 mg/liter, galactomannan was detected after 48 h of incubation for *A. fumigatus*, reflecting the minimal fungicidal action at this concentration (usually observed at 2× the MIC) (13) and the long postantifungal effect observed at 1× the MIC (21), together with a sub-MIC effect, which possibly occurred after amphotericin B concentrations fell below the MIC. The absence of galactomannan production at concentrations >2× the MIC reflects the fungicidal activity amphotericin B demonstrated at time-kill assays (20). For *A. flavus*, galactomannan was detected at C_{max} values of 4.8, 2.4, and 1.2 mg/liter after 48, 24, and 6 h as soon as the concentration fell below the MIC, reflecting the minimal killing and absence of postantifungal effects at ≤4× MIC as previously described (21). Of note, at all three doses, galactomannan production was detected after 4 h despite amphotericin B concentrations being higher than the MIC reflecting the slow inhibitory action of amphotericin B against this species, as previously found (22). Finally, the modest delay in galactomannan production of *A. terreus* at all doses reflects the lack of killing, postantifungal and possibly sub-MIC effect and the slow inhibitory action against this species. Thus, the single-dose pharmacodynamics in the present *in vitro* PK/PD model, where amphotericin B concentrations decrease over time, may reflect concentration- and time-dependent inhibitory and killing activities described by MFC, time-kill, and postantifungal effect assays.

Amphotericin B was for decades the treatment of choice for aspergillosis. Clinical and animal data indicated different drug efficacy against infections caused by various *Aspergillus* species (24). Lack of *in vivo* efficacy, however, was not associated with significantly increased MIC values (3, 25, 26), which remained similar for all three species examined in the present study (13, 27). The results obtained by the new *in vitro* model revealed striking differences in the efficacy of amphotericin B against the three *Aspergillus* species despite their similar MICs with the following order: *A. fumigatus* > *A. flavus* > *A. terreus*. These findings are in agreement with previous comparative animal studies where treatment with amphotericin B was more effective against experimental infection caused by *A. fumigatus* than against infection with *A. flavus* and less effective against infection with *A. terreus* (4, 20). In particular, amphotericin B treatment of guinea pigs infected with an *A. flavus* or an *A. fumigatus* strain (each with an MIC of 1 mg/liter), resulted in 0 and 80% survivals, respectively, at the highest dosage of 2.5 mg/kg (4, 20). Furthermore, the *in vivo* PKPD parameter C_{max}/MIC ratio associated with near-maximum survival in an animal model of experimental aspergillosis by *A. fumigatus* was previously found to be 2.4, similar to the C_{max}/MIC ratio found in the present study to be associated with the maximum suppressive effect of amphotericin B against *A. fumigatus* (28). However, differences in pathogenesis and virulence among these species may confound *in vitro-in vivo* correlation (29, 30). Clinical studies also demonstrated a higher mortality rate of infections by *A. terreus* compared to those by *A. fumigatus* despite amphotericin B therapy (31, 32). It seems that the new *in vitro* model, described here, may better characterize the pharmacodynamic characteristics of amphotericin B against the most clinically sig-

nificant *Aspergillus* species than conventional *in vitro* susceptibility systems.

In summary, the *in vitro* model simulated well amphotericin B human pharmacokinetics and demonstrated a differential *in vitro* activity against the three *Aspergillus* species that was not reflected by their respective MICs. The effects observed in the *in vitro* PK/PD model may be the sum of concentration- and time-dependent inhibitory/killing activities exerted by amphotericin B with the greatest activity found against *A. fumigatus* and the lowest against *A. terreus*. Future studies should focus on testing larger collections of isolates in order to describe the distribution of this new pharmacodynamic effect and taking into account protein binding and amphotericin B disposition in human body in order to obtain clinically relevant drug exposures that were not obtained with the current model. A composite pharmacodynamic effect that describes the different *in vitro* activities of amphotericin B may overcome the MIC clustering, assess better antifungal activity, and help distinguish susceptible and from resistant strains.

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