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# Susceptibility Breakpoints for Amphotericin B and *Aspergillus* Species in an *In Vitro* Pharmacokinetic-Pharmacodynamic Model Simulating Free-Drug Concentrations in Human Serum

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Although conventional amphotericin B was for many years the drug of choice and remains an important agent against invasive aspergillosis, reliable susceptibility breakpoints are lacking. Three clinical *Aspergillus* isolates (*Aspergillus fumigatus*, *Aspergillus flavus*, and *Aspergillus terreus*) were tested in an *in vitro* pharmacokinetic-pharmacodynamic model simulating the biphasic 24-h time-concentration profile of free amphotericin B concentrations in human serum with free peak concentrations ( $fC_{max}$ ) of 0.1, 0.3, 0.6, 1.2, and 2.4 mg/liter administered once daily. Drug concentrations were measured with a bioassay, and fungal growth was monitored for 72 h with galactomannan production. The  $fC_{max}/MIC$  corresponding to half-maximal activity ( $P_{50}$ ) was determined for each species, and the percentage of target attainment was calculated for different MICs for the standard (1 mg/kg of body weight) and a lower (0.6-mg/kg) dose of amphotericin B with Monte Carlo simulation analysis. The  $fC_{max}/MICs$  (95% confidence intervals) corresponding to  $P_{50}$  were 0.145 (0.133 to 0.158), 0.371 (0.283 to 0.486), and 0.41 (0.292 to 0.522) for *A. fumigatus*, *A. flavus*, and *A. terreus*, respectively. The median percentages of  $P_{50}$  attainment were  $\geq 88\%$ , 47%, and 0% for *A. fumigatus* isolates with MICs of  $\leq 0.5$ , 1, and  $\geq 2$  mg/liter, respectively, and  $\geq 81\%$ , 24%, and 0% and  $\geq 75\%$ , 15%, and 0% for *A. flavus* and *A. terreus* isolates with MICs of  $\leq 0.25$ , 0.5, and  $\geq 1$  mg/liter, respectively. The lower dose of 0.6 mg/kg would retain efficacy for *A. fumigatus*, *A. flavus*, and *A. terreus* isolates with MICs of  $\leq 0.25$ ,  $\leq 0.125$ , and  $\leq 0.125$  mg/liter, respectively. The susceptibility, intermediate susceptibility, and resistance breakpoints of  $\leq 0.5$ , 1, and  $\geq 2$  mg/liter for *A. fumigatus* and  $\leq 0.25$ , 0.5, and  $\geq 1$  mg/liter for *A. flavus* and *A. terreus* were determined for conventional amphotericin B with a pharmacokinetic-pharmacodynamic model simulating free-drug serum concentrations.

Invasive aspergillosis is a life-threatening disease among patients with leukemia and bone marrow and solid-organ transplantation (1). *Aspergillus fumigatus* is the most common pathogen causing invasive aspergillosis, accounting for  $>70\%$  of the cases, followed by *Aspergillus flavus*, *Aspergillus terreus*, and *Aspergillus niger* (2). Conventional amphotericin B was for many years the drug of choice and is still used against invasive aspergillosis. Despite its *in vitro* potent antifungal activity against *Aspergillus* spp., clinical trials have shown that conventional amphotericin B therapy is associated with  $<60\%$  survival in patients with invasive aspergillosis (3–6). Although underlying disease, immunosuppression, toxicity, and timing of antifungal therapy affect the mortality of these infections, pathogen susceptibility and drug serum concentrations also represent important contributing factors. *In vivo* studies have shown that the best efficacy was achieved when the maximum concentration of amphotericin B in serum exceeded 2.4 times the MIC of the *A. fumigatus* isolate (7). However, *in vitro* antifungal susceptibility testing of amphotericin B is challenged by the lack of reliable susceptibility breakpoints for each *Aspergillus* species.

The epidemiological cutoff values 2, 2, and 4 mg/liter for the CLSI method and 1, 4, and 4 mg/liter for the EUCAST method were determined for *A. fumigatus*, *A. flavus*, and *A. terreus*, respectively, in order to detect isolates with extreme MICs (8, 9). The current susceptibility breakpoints for *Aspergillus* spp. with the CLSI and EUCAST methodologies are  $\leq 1$  mg/liter (9, 10). However, the supporting clinical and experimental *in vivo* data are poor, and no pharmacokinetic-pharmacodynamic (PK-PD) stud-

ies have validated this breakpoint (9). In a retrospective case-control study of 29 patients, 22/23 patients with isolate MICs of  $\geq 2$  mg/liter died, whereas 6/6 patients with isolate MICs of  $\leq 1$  mg/liter survived (11). Of note, 5/6 survivors with isolate MICs of  $\leq 1$  mg/liter underwent surgery or resolved their neutropenia. In two other large retrospective studies of 160 and 40 patients, no correlation was found between an *Aspergillus* MIC of  $\geq 2$  mg/liter and clinical outcome in patients with invasive aspergillosis treated with conventional amphotericin B (12, 13). Notably, more than 95% of *A. fumigatus* isolates have MICs of  $\leq 1$  mg/liter (8). Therefore, the majority of the isolates would be considered wild-type susceptible based on the 1-mg/liter cutoff, while conventional amphotericin B therapy has been associated with  $<60\%$  of survival in all clinical trials (3, 4, 14). Furthermore, treatment failure was observed in patients infected with *A. fumigatus* and *A. flavus* isolates with CLSI MICs of 0.25 to 0.5 mg/liter and 1 mg/liter, respectively, when treated with the standard dose of 1 mg/kg of conventional amphotericin B (15, 16). Thus, a clinically relevant and

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useful susceptibility breakpoint for amphotericin B and *Aspergillus* spp. is warranted.

An *in vitro* pharmacokinetic-pharmacodynamic model was recently developed for *Aspergillus* spp. simulating pharmacokinetics of antifungal drugs (17). This model revealed considerable pharmacodynamic differences among *Aspergillus* isolates reflecting differences in time- and concentration-dependent inhibitory and fungicidal activity that were not captured by the MIC (18). Using the same model and simulating multidose human pharmacokinetics of voriconazole, *in vitro* data were predictive of experimental animal data and clinical outcome for *A. fumigatus*, enabling the determination of susceptibility breakpoints and target values for therapeutic drug monitoring (19).

Therefore, in the present study, we simulated multidose pharmacokinetics of amphotericin B and studied its activity against *A. fumigatus*, *A. flavus*, and *A. terreus*. The *in vitro* PK-PD relationship was described and bridged with human PKs in order to determine susceptibility breakpoints for each species.

## MATERIALS AND METHODS

**Isolates.** Three clinical strains of *A. fumigatus* (AFM 4215; ATCC MYA-1163), *A. flavus* (AFL 113), and *A. terreus* (AT 137) isolated from patients with invasive aspergillosis were studied. The MICs of amphotericin B as determined thrice according to the CLSI broth microdilution method were 1 mg/liter for all *Aspergillus* species. The strains were maintained at  $-70^{\circ}\text{C}$  in 10% glycerol and cultured twice in Sabouraud dextrose agar at  $30^{\circ}\text{C}$  for 5 to 7 days. A conidial suspension was prepared in normal saline with 1% Tween 20. Conidia were counted with a Neubauer chamber in order to obtain a final suspension of  $1 \times 10^3$  CFU/ml, and their concentration was confirmed by quantitative cultures on Sabouraud dextrose agar.

**Antifungal drug and medium.** Pure powder of amphotericin B (Sigma-Aldrich, Bioline, Athens, Greece) was reconstituted at 5,000 mg/liter in dimethyl-sulfoxide and was stored at  $-70^{\circ}\text{C}$ . The medium contained 10.4 g/liter RPMI 1640 with glutamine without sodium bicarbonate (Sigma-Aldrich, St. Louis, MO) and 0.165 M buffer morpholinepropanesulfonic acid (MOPS) (Invitrogen, Carlsbad, CA), pH 7.0, with 100 mg/liter chloramphenicol (Sigma-Aldrich, St. Louis, MO).

***In vitro* pharmacokinetic-pharmacodynamic model.** The *in vitro* pharmacokinetic simulation model consisted of (i) a glass beaker containing 700 ml medium (external compartment [EC]) in which was placed (ii) a dialysis tube of a 10-ml volume (internal compartment [IC]) made of a permeable cellulose membrane, which allowed free diffusion of molecules with a molecular mass of  $<20$  kDa, and (iii) a peristaltic pump (Minipuls Evolution, Gilson, France), which removed the content of EC and added fresh medium within it at a rate equivalent to drug removal from human serum (17). The conidial suspension was inoculated in the IC, where it remained trapped together with the galactomannan (molecular mass of 20 kDa to 60 kDa) produced by the growing mycelia, while nutrients and drug diffused freely between the IC and EC. The concentration of the galactomannan increased with fungal growth, as found previously (20). After inoculation of the IC, the drug was injected into the EC and IC (for rapid equilibration between the two compartments) every 24 h, and its concentration declined over time, with a half-life ( $t_{1/2}$ ) observed in human serum after intravenous administration of amphotericin B. Because amphotericin B degrades *in vitro* at  $37^{\circ}\text{C}$ , concentrations were adjusted to account for the higher clearance. The EC was covered with aluminum foil in order to minimize light exposure and placed on a heated magnetic stirrer ( $37^{\circ}\text{C}$ ). At the beginning of, during, and at the end of each experiment, temperature and flow rate were measured to ensure that they were at the expected values. All experiments were repeated twice.

**Bioassay.** The drug levels in the IC were determined by a microbiological agar diffusion method using the strain *Paecilomyces variotii* ATCC 22319 (18, 21). Specifically, *P. variotii* conidia at final concentration of  $5 \times$

$10^4$  CFU/ml were inoculated into prewarmed ( $54^{\circ}\text{C}$ ) RPMI 1640 medium and MOPS with 15 g/liter agar and poured onto 10- by 10-cm plastic plates. After solidification of the agar, 1-cm-diameter holes were opened and filled with 100  $\mu\text{l}$  of known drug dilutions (range of 0.1 to 8 mg/liter), as well as 100  $\mu\text{l}$  of IC samples. The plates were incubated at  $37^{\circ}\text{C}$  for 24 h, when 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. The diameters of inhibition zones correlated linearly with amphotericin B concentrations ( $R^2 > 0.9$ ). The lowest limit of detection was 0.1 mg/liter, and the inter- and intraday variations were  $<10\%$  within the detection range.

**Pharmacokinetic analysis.** The time-concentration profile of free amphotericin B observed in human serum after intravenous administration of 0.6 and 1 mg/kg/day (22, 23) was simulated in the *in vitro* model. Thus, the free peak concentrations ( $fC_{\text{max}}$ ) of 0.1, 0.3, 0.6, 1.2, and 2.4 mg/liter were introduced in the system once daily for 3 days (for *A. flavus* and *A. terreus*, the  $fC_{\text{max}}$  of 0.8 and 1 mg/liter were also tested in order to differentiate their pharmacodynamics). Although  $fC_{\text{max}}$  of  $>1$  mg/liter are not clinically achievable in human serum because of the drug's solubility limitation, these concentrations were simulated in order to fully describe the *in vitro* exposure-effect relationship. The biphasic 24-h time-concentration profiles of amphotericin B with an alpha phase with a short half-life of  $<1$  h observed within 2 h after drug administration followed by a beta phase with a longer half-life of 6 to 10 h observed 2 to 24 h after drug administration were simulated. Amphotericin B concentrations were determined at 0 h, 4 h, 6 h, 8 h, 20 h, 24 h, 44 h, 48 h, and 72 h after the introduction of the drug in the IC using the bioassay. The data were analyzed with nonlinear regression based on a two-compartment model described by the equation  $C = C_{\alpha}e^{k_{\alpha}t} + C_{\beta}e^{k_{\beta}t}$ , where  $k_{\alpha}$  and  $k_{\beta}$  are the rate constants,  $C_{\alpha}$  and  $C_{\beta}$  are the  $\gamma$  intercepts for alpha and beta, respectively, and  $C$  is the concentration at a given time  $t$ . The half-lives of alpha and beta phases were calculated using the equations  $t_{1/2,\alpha} = k_{\alpha}/\ln(2)$  and  $t_{1/2,\beta} = k_{\beta}/\ln(2)$ , respectively.

**Determination of fungal growth.** Fungal growth in the IC was assessed in samples of 100  $\mu\text{l}$  at regular time intervals by determining galactomannan production using an enzyme-linked immunosorbent assay (ELISA) (Platelia; Bio-Rad, Athens, Greece). Samples were diluted with 200  $\mu\text{l}$  saline in order to reach the final volume of 300  $\mu\text{l}$  before processing. 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 escaped from the IC.

**Pharmacodynamic analysis.** *In vitro* pharmacodynamics of each amphotericin B dose and *Aspergillus* species were determined based on the GI-time relationship analyzed with the  $E_{\text{max}}$  model:  $E = E_{\text{min}} + E_{\text{max}} \times T^{\gamma}/(T^{\gamma} + T_{50}^{\gamma})$ , where  $E$  is the GI (dependent variable),  $E_{\text{max}}$  and  $E_{\text{min}}$  are the maximum and minimum GI, respectively,  $T$  is the incubation time (independent variable),  $T_{50}$  is the time corresponding to 50% of the  $E_{\text{max}}$ , and  $\gamma$  is the slope of the curve. As shown previously, the parameters  $E_{\text{max}}$ ,  $\gamma$ , and  $T_{50}$  describe the extent, rate, and time of galactomannan production, respectively, whereas the area under the galactomannan index-time curve ( $\text{AUC}_{\text{GI}}$ ) is a surrogate marker of fungal growth capturing any differences in extent, rate, and time of antifungal activity. The higher the  $\text{AUC}_{\text{GI}}$ , the greater is the fungal growth over time. The area under the galactomannan index-time curve ( $\text{AUC}_{\text{GI}}$ ) was calculated for each amphotericin B dose and drug-free control. The percentage of fungal growth at each dose was calculated based on the  $\text{AUC}_{\text{GI}}$  of each dose divided by the  $\text{AUC}_{\text{GI}}$  of the drug-free growth control. The percent growth- $fC_{\text{max}}/\text{MIC}$  relationship was analyzed with the  $E_{\text{max}}$  model:  $E = E_{\text{min}} + E_{\text{max}} \times P^{\gamma}/(P^{\gamma} + P_{50}^{\gamma})$ , where  $E$  is the percent growth (dependent variable),  $E_{\text{max}}$  and  $E_{\text{min}}$  are the maximum and minimum percent growth, respectively,  $P$  is the PK-PD index  $fC_{\text{max}}/\text{MIC}$  (independent variable),  $P_{50}$  is the  $fC_{\text{max}}/\text{MIC}$  corresponding to 50% of  $E_{\text{max}}$ , and  $\gamma$  is the slope of the curve. In addition, the  $fC_{\text{max}}/\text{MIC}$  corresponding to near-maximum an-

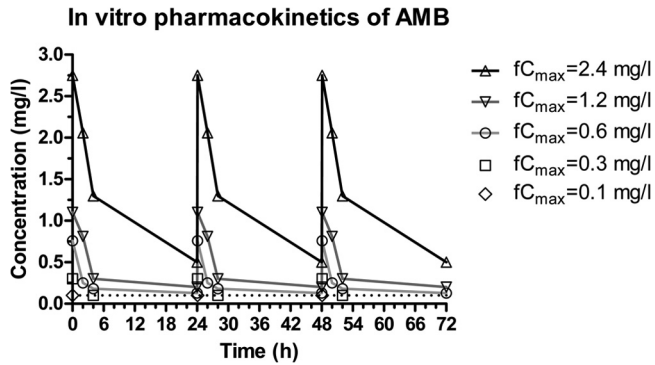


FIG 1 Multidose pharmacokinetics of amphotericin B in the *in vitro* model with different  $fC_{max}$  and  $t_{1/2}$  of 0.2 to 2 h and 10 to 17 h for alpha and beta phases, respectively. Coefficient of variations was  $\pm 15\%$  between two replicates.

tifungal activity (i.e., 80% inhibition or 20% growth;  $P_{80}$ ) was calculated for each *Aspergillus* species. The 95% confidence interval (CI) of the parameters  $P_{50}$  and  $P_{80}$  obtained with the regression analysis was used as an estimate of both biological and experimental variation as previously described (19).

**Monte Carlo simulation.** In order to bridge the *in vitro* data with human PKs, the steady-state  $fC_{max}$  of 1,000 patients receiving the standard dose of amphotericin B, 1 mg/kg intravenously, were simulated with Monte Carlo analysis. This dosage resulted in steady-state total maximum concentrations in human serum ( $tC_{max}$ ) of  $2.83 \pm 1.17$  mg/liter, respectively (23), which corresponds to free maximum concentrations ( $fC_{max}$ ) of  $0.14 \pm 0.06$  mg/liter based on the 95% protein binding rate of amphotericin B previously found at these concentrations (24). The percentage of patients with  $fC_{max}/MIC$  exceeding the median, upper, and lower 95% CI limits of the PK-PD targets  $P_{50}$  and  $P_{80}$  were calculated for different MICs and for each *Aspergillus* species.

Because the dose of 1 mg/kg is associated with significant toxicity, Monte Carlo analysis was also performed for a lower dose of conventional

amphotericin B, 0.6 mg/kg, used in clinical practice. This dose resulted in a serum  $tC_{max}$  of  $1.43 \pm 0.2$  mg/liter, corresponding to an  $fC_{max}$  of  $0.0715 \pm 0.01$  mg/liter (22, 24). The median, upper, and lower 95% CI limits of percentage of  $P_{50}$  target attainment were calculated for each species and for different MICs. Finally, because trough levels ( $C_{min}$ ) are clinically easier obtained than  $C_{max}$  levels, the trough levels were associated using linear regression analysis with  $C_{max}$  concentrations in the *in vitro* PK-PD model.

**Statistical analysis.** All analysis was performed with the Prism 5.01 software (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 among *Aspergillus* species were assessed using the extra sum-of-squares F test. Monte Carlo analysis was performed with the random function of an Excel spreadsheet (MS Office 97). A  $P$  value of  $<0.05$  was considered statistically significant.

**RESULTS**

**Pharmacokinetic analysis.** The time-concentration profiles of amphotericin B doses are shown in Fig. 1. The calculated  $fC_{max}$  in the IC were within  $\pm 20\%$  of the target  $fC_{max}$  of 0.1, 0.3, 0.6, 0.8, 1, 1.2, and 2.4 mg/liter. The half-lives of alpha and beta phases were 0.2 to 2 h and 10 to 17 h, respectively.

**Pharmacodynamic analysis.** Fungal growth was progressively inhibited at higher concentrations of amphotericin B. The GI-time curves followed a sigmoid pattern described well by the  $E_{max}$  model ( $R^2 > 0.82$ ), and they were characterized by the same  $E_{max}$  but different slopes and  $T_{50s}$  for the different doses of amphotericin B and *Aspergillus* species tested (Fig. 2). Complete inhibition of fungal growth was found for *A. fumigatus* with an amphotericin B  $fC_{max}$  of  $\geq 0.3$  mg/liter, while for *A. flavus* and *A. terreus*, complete inhibition was obtained with an amphotericin B  $fC_{max}$  of  $\geq 0.8$  mg/liter. In particular, the percentages of fungal growth at  $fC_{max}$  of 0.1, 0.3, and 0.6 mg/liter were 97%, 78%, and 74% for *A. flavus* and 96%, 82%, and 67% for *A. terreus*, respectively.

The  $fC_{max}/MIC$  relationship followed a sigmoid pattern (global  $R^2 = 0.96$ ) for all three species (Fig. 3). The  $fC_{max}/MIC$  (95% CI)

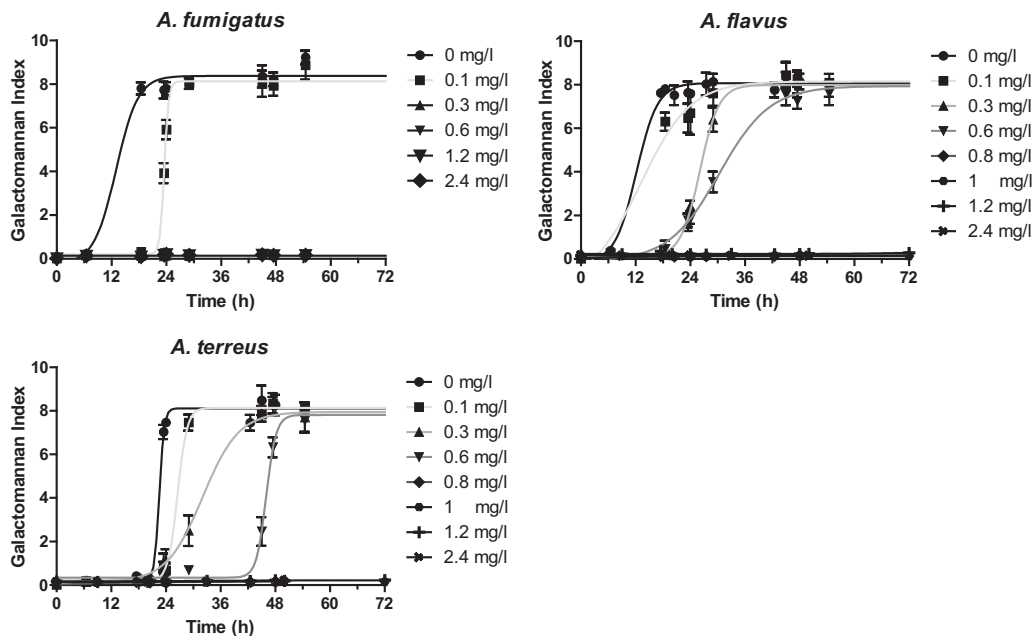


FIG 2 Multidose pharmacodynamic analysis of simulated amphotericin B doses with  $fC_{max}$  of 0.1, 0.3, 0.6, 0.8, 1, 1.2, and 2.4 mg/liter against *A. fumigatus*, *A. flavus*, and *A. terreus* isolates with a modal CLSI MIC of 1 mg/liter as determined by the galactomannan index in the *in vitro* PK-PD model.



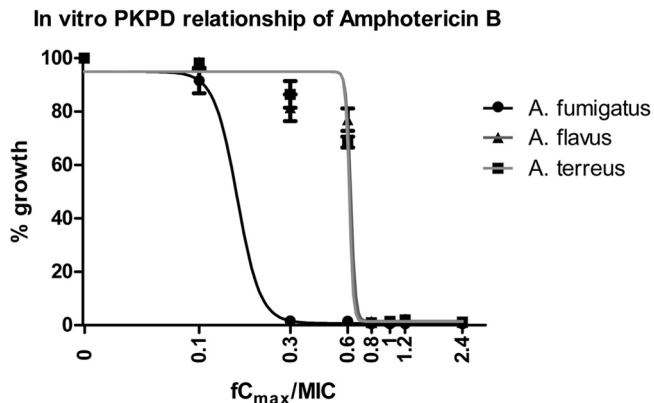


FIG 3 Multidose exposure-efficacy relationship of amphotericin B against each *Aspergillus* species with modal CLSI MICs of 1 mg/liter for *A. fumigatus*, *A. flavus*, and *A. terreus* in the *in vitro* PK-PD model simulating amphotericin B human serum levels based on the increasing amphotericin B  $fC_{max}$  (maximum concentration) and the galactomannan index as a marker of fungal growth.

associated with 50% growth for *A. fumigatus* was 0.145 (0.133 to 0.158), which was statistically significantly lower than the corresponding  $fC_{max}/MIC$  of *A. flavus* (0.371; 0.283 to 0.486) and *A. terreus* (0.41; 0.292 to 0.522) ( $P < 0.001$ ). The  $fC_{max}/MIC$ s corresponding to near maximal activity (20% growth) were 0.188 (0.166 to 0.213), 0.474 (0.362 to 0.655), and 0.545 (0.376 to 0.714) for *A. fumigatus*, *A. flavus*, and *A. terreus*, respectively.

**Monte Carlo simulation analysis.** Monte Carlo simulation analysis of 1,000 patients receiving the standard amphotericin B dose of 1 mg/kg resulted in mean  $tC_{max} \pm$  standard deviation (SD) values of  $2.8 \pm 1.2$  mg/liter ( $fC_{max} \pm$  SD of  $0.14 \pm 0.06$  mg/liter). These values are close to the target values previously found in clinical studies (23). The percentage of patients attaining the PK-PD target ( $fC_{max}/MIC$ ) associated with half-maximal ( $P_{50}$ ) activity is shown in Fig. 4. Higher-than-75% and lower-than-15%  $P_{50}$  target attainment was found for *A. fumigatus* isolates with MICs of  $\leq 0.5$  and  $\geq 2$  mg/liter and for *A. flavus* and *A. terreus* isolates with MICs of  $\leq 0.25$  and  $\geq 1$  mg/liter. In particular, the median (range) percentages of target attainment for *A. fumigatus*, *A. flavus*, and *A. terreus* isolates with a MIC of 1 mg/liter were 47% (39 to 57%), 0% (0 to 0%), and 0% (0 to 0%) and with a MIC of 0.5 mg/liter were 88% (87 to 90%), 24% (5 to 50%), and 15% (2 to

46%), respectively. The percentages of  $P_{80}$  target attainment for *A. fumigatus*, *A. flavus*, and *A. terreus* isolates with a MIC of 1 mg/liter were 22%, 0%, and 0%, respectively (data not shown).

For the lower amphotericin B dose of 0.6 mg/kg, a  $>98\%$   $P_{50}$  target attainment was found for MICs of  $\leq 0.25$ ,  $\leq 0.125$ , and  $\leq 0.125$  mg/liter for *A. fumigatus*, *A. flavus*, and *A. terreus*, respectively (Fig. 4). A linear correlation ( $r^2 = 0.98$ ) was found between the  $C_{min}$  and the  $C_{max}$  levels in the *in vitro* model with a slope of  $0.20 \pm 0.03$ , indicating that  $C_{min}$  levels correspond to 1/5 of the  $C_{max}$  concentrations, as previously found in humans (22, 23).

## DISCUSSION

The multidose pharmacodynamic study of amphotericin B using an *in vitro* pharmacokinetic-pharmacodynamic model that simulated free serum amphotericin B concentrations revealed differences among the three *Aspergillus* species. Complete growth inhibition was observed at an  $fC_{max}$  of  $\geq 0.3$  mg/liter for *A. fumigatus* and  $\geq 0.8$  mg/liter for *A. flavus* and *A. terreus* despite their identical MIC values. The  $fC_{max}/MIC$ s (95% CI) corresponding to near-maximal activity were 0.188 (0.166 to 0.213), 0.474 (0.362 to 0.655), and 0.545 (0.376 to 0.714) for *A. fumigatus*, *A. flavus*, and *A. terreus*, respectively. These differences are in agreement with our previous study where single-dose pharmacokinetics and a higher initial inoculum were used, providing better discrimination of the *in vitro* activity of amphotericin B against the three species (18). The observed differences among the three species were attributed to the 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* (18).

The PK-PD target of near maximal antifungal activity found in the present study against *A. fumigatus* is in agreement with that previously obtained in a murine neutropenic model of experimental pulmonary aspergillosis demonstrating that the  $tC_{max}/MIC$  of 2.4 was associated with near-maximal survival (7). Considering that the free-drug percentage of conventional amphotericin B in mouse serum corresponded to 7.4% (25), the *in vivo*  $fC_{max}/MIC$  corresponds to 0.178, which is very close to the *in vitro*  $fC_{max}/MIC$  of 0.188 found in the present study. In addition, in neutropenic murine models of experimental aspergillosis, the dose of 5 mg/kg of amphotericin B was associated with  $>80\%$  survival against *A. fumigatus* isolates with MICs of 0.5 and 1 mg/liter and  $<40\%$  against *A. terreus* and *A. flavus* isolates with MICs

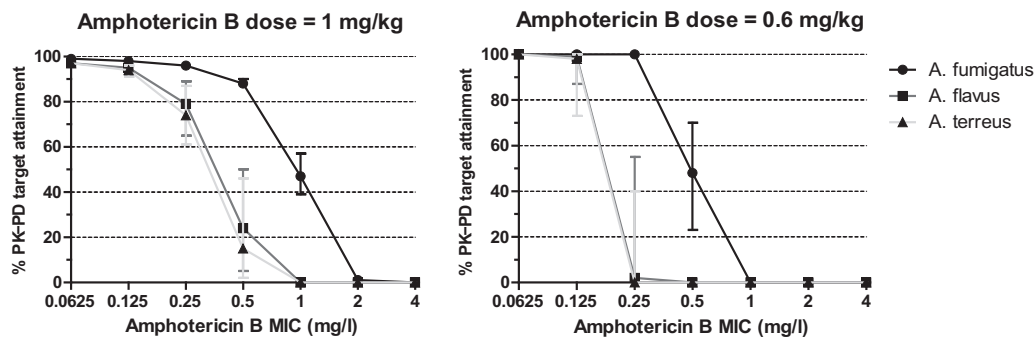


FIG 4 The percentage of patients attaining the PK-PD target associated with half-maximal activity ( $P_{50}$ ) for *A. fumigatus*, *A. flavus*, and *A. terreus* isolates with different MICs based on the Monte Carlo analysis simulating 1,000 patients treated with 1 or 0.6 mg/kg of conventional amphotericin B. Error bars represent the range of percentage of target attainment for the upper and lower 95% CI limit of the  $P_{50}$  target.

of 1 to 2 mg/liter (26 to 29). The dose of 5 mg/kg, which corresponds to a  $tC_{max}$  around 2.5 mg/liter (i.e.,  $fC_{max}$  of 0.185 mg/liter) in mouse serum (7, 30), would attain the PK-PD targets determined in the present study for *A. fumigatus* but not for *A. terreus* and *A. flavus* isolates. However, there are many confounding factors, like immunosuppression, type and severity of infection, dosing regimens, time of initiation of antifungal therapy, pharmacokinetic variation, and *in vitro* susceptibility testing, that prohibited direct *in vitro-in vivo* correlation (31 to 33). These factors can be simulated in the present model in order to study the impact of host defense cells, delayed drug administration, and disease progression on the magnitude of the PK-PD parameter.

The present *in vitro* model indicated that the standard dose of 1 mg/kg of amphotericin B is associated with 47% target attainment using a half-maximum activity PK-PD target ( $P_{50}$ ) and 22% using a near-maximum activity PK-PD target ( $P_{80}$ ) for *Aspergillus fumigatus* isolates with an amphotericin B MIC of 1 mg/liter. This is in agreement with a previous retrospective study where the MICs of 274 clinical *Aspergillus* isolates from transplant recipients with proven or probable invasive aspergillosis were analyzed together with survival after 6 and 12 weeks of treatment (34). In that study, isolates with amphotericin B MICs of 1 mg/liter belonging mainly to *Aspergillus fumigatus* species were associated with 45% 6-week and 25% 12-week survival, in agreement with the percentages of  $P_{50}$  and  $P_{80}$  target attainment found in the present study, respectively.

The choice of the *in vitro* PK-PD target ( $P_{50}$  or  $P_{80}$ ) for correlating *in vitro* data with clinical outcome remains controversial. The percentage of  $P_{80}$  target attainment was comparable to 12-week survival, whereas the percentage of  $P_{50}$  target attainment was comparable to 6-week survival. High serum drug exposure ( $P_{80}$ ) may result in high concentrations at the site of infection and/or fungicidal actions that will likely produce a long-lasting effect (12-week survival) compared to the lower drug exposure ( $P_{50}$ ) that will produce a short-lasting effect (6-week survival). However, since most deaths during the second 6-week period of 12-week therapy were attributed to causes other than invasive aspergillosis, albeit with some uncertainty, the 6-week survival endpoint was suggested to be the preferable endpoint to judge the effectiveness of antifungal therapy (35). Because the percentage of  $P_{50}$  target attainment found by Monte Carlo analysis of the present study for *A. fumigatus* isolates with an amphotericin B MIC of 1 mg/liter was correlated with the 6-week survival found previously in a retrospective study of the MIC-clinical outcome correlation for the same isolates (34), we used the  $P_{50}$  endpoint to bridge the *in vitro* PK-PD data with human PK in Monte Carlo analysis and to determine susceptibility breakpoints.

An important assumption when bridging *in vitro* concentrations to serum PK is that the serum concentrations correspond to the tissue concentrations at the site of infection, which is the lung in the case of invasive pulmonary aspergillosis. *In vivo* pharmacokinetic data of conventional amphotericin B in mice showed that although the  $tC_{max}$  in the lung was lower than the  $tC_{max}$  in serum (3.33 and 4.58 mg/liter, respectively), the  $fC_{max}$  values were similar (0.33 and 0.34 mg/liter, respectively) based on the different degree of protein binding in the lung (10%) compared to that in serum (7.4%) (30). In two clinical studies, a wide range of amphotericin B lung concentrations (0.1 to 23.3  $\mu$ g/g) were detected with high-performance liquid chromatography (HPLC) in patients with mean concentrations of 11.29 and 5.29  $\mu$ g/g (36, 37). However,

only a small (1.5% to 30%) proportion of HPLC-detected amphotericin B was bioactive, and an even smaller proportion of the latter (<10%) corresponded to free amphotericin B (36, 37). Thus, the free concentration of amphotericin B in the lung may be similar to the free drug concentrations in human serum. For other sites of infections where amphotericin B penetrates poorly (e.g., brain), the tissue-to-serum ratio should be taken into account. Microdialysis experiments may provide better estimates of free amphotericin B in tissues (38).

The results obtained by the Monte Carlo analysis point to the following breakpoints for susceptibility, intermediate susceptibility, and resistance:  $\leq 0.5$ , 1, and  $\geq 2$  for *A. fumigatus* and  $\leq 0.25$ , 0.5, and  $\geq 1$  for *A. flavus* and *A. terreus*. Based on these breakpoints and previous epidemiological susceptibility data, 40% of *A. fumigatus*, 90% of *A. flavus*, and 98% of *A. terreus* isolates would be considered nonsusceptible (8). In a randomized prospective clinical trial of invasive aspergillosis mainly by *A. fumigatus*, conventional amphotericin B was associated with 42% 6-week mortality, which is in agreement with the 40% of *A. fumigatus* isolates estimated in the present study being nonsusceptible (3). Furthermore, in a review paper of 60 clinical cases of invasive *A. terreus* infections published from 1966 to 2003, 28 cases were treated with conventional amphotericin B, and the mortality rate was 96% (27/28), which is also in agreement with the 98% of *A. terreus* isolates estimated in the present study being nonsusceptible (39). Finally, in a cohort study of 11 cases of invasive *A. flavus* infections treated only with conventional amphotericin B, the mortality rate was 81% (9/11), in agreement with the 90% of *A. flavus* isolates estimated in the present study being nonsusceptible (16). Interestingly, in the latter study, all patients with *A. flavus* isolate MICs of  $\geq 1$  mg/liter died. These clinical data provide further support of the breakpoints determined in the present PK-PD model.

In order to maximize the efficacy and minimize the toxicity of conventional amphotericin B treatment, one could increase or decrease the dose based on the MIC of isolated pathogens or epidemiological data of each center. Thus, for a small number of *A. fumigatus*, *A. flavus*, and *A. terreus* strains with MICs of  $\leq 0.25$ ,  $\leq 0.125$ , and  $\leq 0.125$  mg/liter, respectively, the lower dose of 0.6 mg/kg would retain efficacy of the 1-mg/kg dosage but reduce its toxicity. The 0.6-mg/kg dose was associated with 30% renal toxicity compared to >50% renal toxicity associated with the 1-mg/kg dose of amphotericin B (5, 40). For isolates with intermediate susceptibility, therapeutic drug monitoring (TDM) with the goal to attain the PK-PD target could increase the efficacy of the standard dose of 1 mg/kg of amphotericin B as previously suggested (41). Since TDM is easier performed using trough ( $C_{min}$ ) levels than  $C_{max}$  levels, the  $fC_{min}/MIC$  ( $tC_{min}/MIC$ ) ratios based on the *in vitro* model were 0.029 (0.58), 0.074 (1.48), and 0.082 (1.64) for *A. fumigatus*, *A. flavus*, and *A. terreus*, respectively. Since *in vivo* amphotericin B is bound by tissue and slowly released, accounting for the prolonged (serum) terminal elimination half-life of the drug, the correlation between  $fC_{min}$  and  $fC_{max}$  may be clinically relevant only for the first few days of therapy. However, the unavoidable toxicity of conventional amphotericin B, which often results in drug discontinuation, will be the limiting factor even for susceptible isolates, limiting the use of conventional amphotericin B, in agreement with the ECIL guidelines for the management of invasive aspergillosis (42). Therefore, lipid formulations of amphotericin B could be used efficiently even for isolates with reduced susceptibility to conventional amphotericin

B, as previously shown in an animal model infected by an *A. fumigatus* isolate with a MIC of 1 mg/liter (43). Lipid formulations can safely deliver high concentrations of amphotericin B in the fungal target and at the site of infection (44).

In conclusion, a PK-PD model was used to simulate free amphotericin B serum concentrations and to study the *in vitro* activity against three *Aspergillus* species. The PK-PD targets were 0.145, 0.371, and 0.41  $fC_{max}/MIC$  for *A. fumigatus*, *A. flavus*, and *A. terreus*, respectively, and the susceptibility breakpoints derived were  $\leq 0.5$ , 1, and  $\geq 2$  mg/liter for *A. fumigatus* and  $\leq 0.25$ , 0.5, and  $\geq 1$  mg/liter for *A. flavus* and *A. terreus*. The suggested breakpoints are substantiated with results obtained from previously published clinical studies of invasive aspergillosis.

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