AAC
Journals.ASM.org

# Pharmacodynamic Effects of Simulated Standard Doses of Antifungal Drugs against *Aspergillus* Species in a New *In Vitro* Pharmacokinetic/Pharmacodynamic Model

**Joseph Meletiadis,[a] Rafal Al-Saigh,[a] Aristea Velegraki,[b] Thomas J. Walsh,[c] Emmanuel Roilides,[d] and Loukia Zerva[a]**

Clinical Microbiology Laboratory, Attikon University Hospital, Medical School, National and Kapodistrian University of Athens, Athens, Greece[a]; Mycology Laboratory, Department of Microbiology, Medical School, National and Kapodistrian University of Athens, Athens, Greece[b]; Transplantation-Oncology Infectious Diseases Program, Division of Infectious Diseases, Weill Medical College of Cornell University and New York Presbyterian Hospital, New York, New York[c]; and Infectious Diseases Unit, 3rd Department of Pediatrics, Aristotle University School of Medicine, Hippokration Hospital, Thessaloniki, Greece[d]

**In conventional MIC tests, fungi are exposed to constant drug concentrations, whereas *in vivo*, fungi are exposed to changing drug concentrations. Therefore, we developed a new *in vitro* pharmacokinetic/pharmacodynamic model where human plasma pharmacokinetics of standard doses of 1 mg/kg amphotericin B, 4 mg/kg voriconazole, and 1 mg/kg caspofungin were simulated and their pharmacodynamic characteristics were determined against three clinical isolates of *Aspergillus fumigatus*, *Aspergillus flavus*, and *Aspergillus terreus* with identical MICs (1 mg/liter for amphotericin B, 0.5 mg/liter for voriconazole) and minimum effective concentrations (0.5 mg/liter for caspofungin). This new model consists of an internal compartment (a 10-ml dialysis tube made out of a semipermeable cellulose membrane allowing the free diffusion of antifungals but not galactomannan) inoculated with *Aspergillus* conidia and placed inside an external compartment (a 700-ml glass beaker) whose content is diluted after the addition of antifungal drugs by a peristaltic pump at the same rate as the clearance of the antifungal drugs in human plasma. Fungal growth was assessed by galactomannan production. Despite demonstrating the same MICs, amphotericin B completely inhibited (100%) *A. fumigatus* but not *A. flavus* and *A. terreus*, whose growth was delayed for 7.53 and 22.8 h, respectively. Voriconazole partially inhibited *A. fumigatus* (49.5%) and A. *flavus* (27.9%) but not A. *terreus*; it delayed their growth by 3.99 h (*A. fumigatus*) and 5.37 h (A. *terreus*). Caspofungin did not alter galactomannan production in all of the species but *A. terreus*. The new model simulated human pharmacokinetics of antifungal drugs and revealed important pharmacodynamic differences in their activity.**

Invasive aspergillosis is associated with high morbidity and mortality, particularly in patients with hematologic malignancies or undergoing bone marrow transplantation. Among the most common *Aspergillus* species implicated in these severe infections are *Aspergillus fumigatus*, *Aspergillus flavus*, and *Aspergillus terreus* (19, 24). *In vivo* experimental and clinical data show that the efficacy of various antifungal agents differs for these species (31, 35). However, standardized *in vitro* susceptibility testing of these three species against amphotericin B, voriconazole, and caspofungin demonstrates comparable/similar activities of these agents against the vast majority of isolates, making *in vitro-in vivo* activity correlation difficult (18, 20).

The *in vitro* activities of antifungal agents against *Aspergillus* spp. are determined by standardized broth microdilution assays where the MIC (for azoles and amphotericin B) or minimum effective concentration (MEC; for echinocandins) is defined as the lowest drug concentration that completely inhibits fungal growth or causes typical morphological changes in the fungus, respectively (5). These endpoints are obtained using assays where the drug concentration remains constant over time (4). However, fungi are exposed *in vivo* to fluctuating drug concentrations over time because of absorption, elimination, excretion, metabolism, and distribution processes (9). The impact of fluctuating drug concentrations on *Aspergillus* growth is unknown and might be different from the effect of constant drug concentrations over time (16). Furthermore, a variety of important pharmacokinetic/pharmacodynamic (PK/PD) indices cannot be studied *in vitro* against *Aspergillus* spp. since MIC testing by standard methodology does not produce clinically relevant drug exposures (1).

*In vitro* models that simulate the human plasma pharmacokinetics of antifungal drugs have been developed for *Candida* species, enabling the study of the effects of clinically relevant drug concentrations on different *Candida* species (13, 15). Furthermore, they have proved to be very useful in investigating the efficacy of antifungal drug combinations against *Candida* spp. (14, 17). The development of similar models for *Aspergillus* spp. has been hampered so far by their filamentous growth, which makes it difficult to control drug exposure and determine fungal growth.

The purposes of this study were to develop an *in vitro* model that simulates the pharmacokinetics of amphotericin B, voriconazole, and caspofungin in humans after the intravenous administration of standard doses and to study the effects of these antifungal agents on clinical isolates of *A. fumigatus*, *A. flavus*, and *A. terreus*.

Received 15 May 2011 Returned for modification 16 July 2011
Accepted 31 October 2011
Published ahead of print 7 November 2011
Address correspondence to Joseph Meletiadis, jmeletiadis@med.uoa.gr.

doi:10.1128/AAC.00662-11

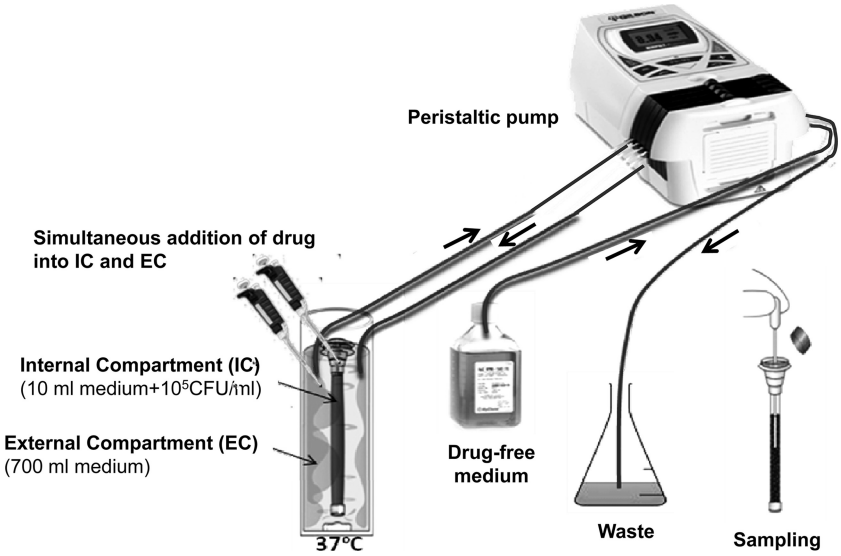


**FIG 1** Development of an *in vitro* PK/PD model that simulates the pharmacokinetics of antifungal drugs in human plasma and determines a drug's effect on *Aspergillus* growth. The model consists of an EC, a glass beaker containing 700 ml broth medium, and an IC, a dialysis tube containing 10 ml broth medium; the tube is made of a semipermeable cellulose membrane that allows free passage of small molecules (<20 kDa) like antifungals, but not galactomannan. The EC is placed on a heated magnetic stirrer (37°C and 2 rpm). A peristaltic pump introduces drug-free medium into the EC and removes its content concurrently in order to maintain a constant volume. The flow rate is adjusted to achieve drug concentrations corresponding to the drug's clearance from human plasma. At time zero, $10^5$ CFU/ml of *Aspergillus* conidia are inoculated into the IC, while the drug is introduced into both the EC and the IC for rapid concentration equilibration. Subsequently, the drug concentration declines over time. Galactomannan levels in the IC medium are measured at regular time points.

## MATERIALS AND METHODS

**Isolates.** Three isolates of *A. fumigatus*, *A. flavus*, and *A. terreus* obtained from patients with invasive pulmonary aspergillosis were used. By the CLSI M38A2 reference method (5), the MICs were 1 mg/liter for amphotericin B and 0.5 mg/liter for voriconazole, whereas the caspofungin MEC was 0.5 mg/liter tested in triplicate. The isolates were maintained at −70°C in 10% glycerol and were subcultured twice in Sabouraud dextrose agar at 30°C for 5 to 7 days before testing. Conidial suspensions were prepared in normal saline with 1% Tween 20. The inoculum concentration ($1 \times 10^5$ CFU/ml) was determined with a Neubauer chamber and by culturing serial dilutions on Sabouraud dextrose agar.

**Antifungal drugs.** Amphotericin B (Fungizone; molecular mass, 0.92 kDa; Bristol-Myers, Athens, Greece), voriconazole (Vfend; molecular mass, 0.35 kDa; Pfizer, Athens, Greece), and caspofungin (Cancidas; molecular mass, 1.1 kDa; Merck, Athens, Greece) were reconstituted at 5,000 mg/liter, 10,000 mg/liter, and 5,000 mg/liter, respectively, according to the manufacturers' instructions and stored at −70°C. Preliminary studies with pure drugs did not show any differences in fungal growth inhibition compared to commercial formulations. Therefore, all further studies were performed with commercial formulations.

**Medium.** The medium used throughout this study contained 10.4 g/liter RPMI 1640 with glutamine without sodium bicarbonate (Sigma-Aldrich, St. Louis, MO) and 0.165 M morpholinepropanesulfonic acid buffer (Invitrogen, Carlsbad, CA), pH 7.0, with 100 mg/liter chloramphenicol (Sigma-Aldrich, St. Louis, MO).

***In vitro* PK/PD model.** The *in vitro* PK/PD simulation model used in this study is shown in Fig. 1. It consists of a 10-ml-volume dialysis tube (internal compartment [IC]) made of a semipermeable cellulose membrane (Float-A-Lyzer; Spectrum Europe B.V., Breda, The Netherlands) allowing the free diffusion of small molecules (molecular mass, <20 kDa). This is placed in a glass beaker containing 700 ml medium (external compartment [EC]) the content of which is continuously diluted by a peristaltic pump (Minipuls Evolution; Gilson Inc., Villiers le Bel, France) removing drug-containing medium from the EC and adding drug-free medium to the EC at a rate equivalent to drug clearance from human plasma.

At time zero, the IC was inoculated with 10 ml medium containing $1 \times 10^5$ CFU/ml of *Aspergillus* conidia. The cellulose membrane of the IC allowed the free diffusion of nutrients and drugs until an equilibrium with the EC was reached, while at the same time it retained the conidia, hyphae, and macromolecular products such as galactomannan (molecular mass of 25 to 75 kDa). Thus, galactomannan was concentrated inside the IC and was used as a biomarker of fungal growth. In order to ensure that galactomannan did not diffuse into the EC, galactomannan levels were determined in the EC.

Also at time zero, the drug was injected into the EC and the IC simultaneously in order to achieve rapid equilibration of the drug concentrations in the two compartments (preliminary experiments showed that when the drug was added only to the EC, there was a lag of 4 h until an equilibrium was reached). The drug-containing medium in the EC was then continuously diluted with drug-free medium by the peristaltic pump adjusted to a specific flow rate in order to reproduce average drug half-lives ($t_{1/2}$s) observed in human plasma after the intravenous administration of amphotericin B (19 h), caspofungin (12 h), and voriconazole (6 h) in accordance with previous clinical studies (2, 28, 34). The EC is then placed on a heated magnetic stirrer adjusted to 37°C and 2 rpm. The temperature and flow rate were checked regularly throughout the experiment using a thermometer and by measuring the volume of medium pumped out of the EC in 1 min, respectively.

**Bioassays for determination of drug levels.** The drug levels in the EC and the IC were determined by microbiological methods using *Candida kefyr* strain NCPF 3234 for voriconazole and caspofungin and *Paecilomyces variotii* strain ATCC 22319 for amphotericin B as described previously for amphotericin B and voriconazole (25, 30). Briefly, $3 \times 10^5$ CFU/ml *C. kefyr* or $5 \times 10^4$ CFU/ml *P. variotii* were inoculated into prewarmed medium (54°C) containing 15 g/liter agar (Difco Bacto Agar; BD Hellas SA, Athens, Greece). The medium was poured into square (10 by 10 cm) plastic petri dishes, and after solidification, 2-mm-diameter holes were opened. One-hundred-microliter serial 2-fold drug dilutions (range, 0.25 to 16 mg/liter) and 100-$\mu$l samples obtained from the EC or IC medium were added to the holes. The plates were incubated at 37°C for 24 h, and the diameters of the inhibition zones around the holes were measured with a ruler. A standard curve of drug concentrations versus diameters of

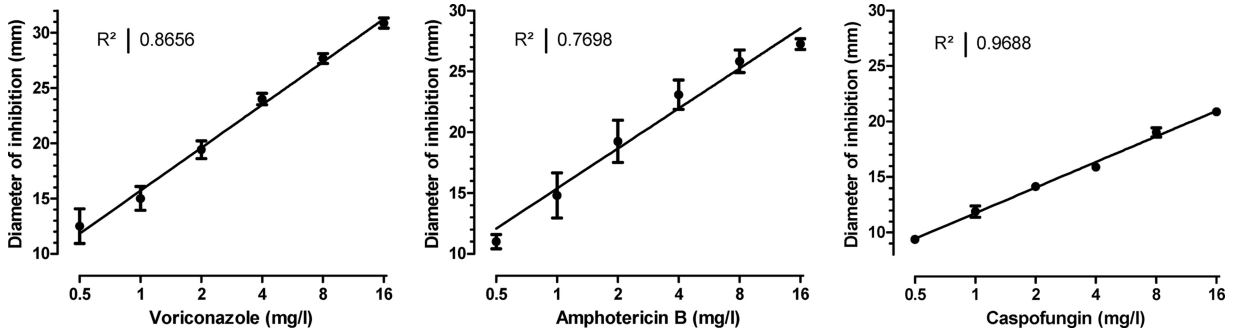


**FIG 2** Validation of bioassays used for the determination of amphotericin B, voriconazole, and caspofungin concentrations. The correlation between diameter of inhibition zones and drug concentrations is shown together with the respective correlation coefficients of linear regression analysis. Results were obtained from eight experiments for each drug on different days. Error bars represent standard deviations.

inhibition zones was constructed and subjected to linear regression analysis. Based on this standard curve, the drug concentrations of the EC and the IC were determined at any time point. In order to ensure that the drug concentration within the IC was uniform, samples from the center and periphery of the top, middle, and bottom parts of the IC were tested.

**Pharmacokinetic analysis.** The following standard doses were simulated in the *in vitro* model: 1 mg/kg amphotericin B, 1 mg/kg caspofungin, and 4 mg/kg voriconazole with average peak total concentrations in human plasma of 2.8, 10, and 3.6 mg/liter and average $t_{1/2}$s of 19 h, 12 h, and 6 h, respectively, after intravenous administration at steady state (2, 28, 34). The levels of antifungal drugs at 0, 4, 6, 8, 20, and 24 h after the introduction of the drug were determined in the IC and EC with the bioassays. The data were subjected to nonlinear regression analysis based on the one-compartment pharmacokinetic model, which is described by the equation $C_t = C_0 e^{-k/t}$, where $C_t$ (dependent variable) is the drug concentration at a given time (independent variable), $C_0$ the initial drug concentration at 0 h, $e$ is the physical constant 2.18, and $k$ is the rate of drug removal. The $t_{1/2}$ was calculated using the equation $t_{1/2} = k/0.693$ for the EC and the IC separately and compared with the respective values obtained in human plasma (2, 28, 34) using Student's $t$ test. Finally, the area under the concentration-time curve from 0 to 24 h after dose administration ($AUC_{0\text{-}24}$) was calculated by the trapezoidal rule.

**Galactomannan levels for determination of fungal growth.** Fungal growth in the IC was monitored by galactomannan production. Galactomannan levels were measured by enzyme-linked immunosorbent assay (Platelia; Bio-Rad Laboratories, Athens, Greece), and results were expressed as a galactomannan index (GI) according to the manufacturer's instructions. In order to correlate GIs with fungal growth, different IC tubes were inoculated with $10^3$, $10^4$, $10^5$, or $10^6$ CFU/ml *A. fumigatus* and incubated without a drug for 24 h at 37°C in the *in vitro* PK/PD model and GIs were determined at regular time intervals. The kinetics of the GIs were subjected to nonlinear regression analysis based on the $E_{max}$ model described by the equation $E = E_{max} \cdot T^{\gamma}/(T^{\gamma} + T_{50})$, where $E$ is the GI (dependent variable), $E_{max}$ is the maximum GI, $T$ is the incubation time (independent variable), $T_{50}$ is the time corresponding to 50% of $E_{max}$, and $\gamma$ is the slope of the curve. The $T_{50}$ parameter was then correlated with the initial concentration of the suspension of conidia by linear regression analysis.

Galactomannan levels were also correlated with real-time PCR results. IC tubes were inoculated with increasing inocula of *A. fumigatus*, and DNA was extracted from 100-$\mu$l samples with a Maxwell 16-cell DNA purification kit according to the manufacturer's instructions (Promega, Madison, WI) after incubation for 2, 4, 8, and 24 h at 37°C in the *in vitro* PK/PD model. Real-time PCR was performed with the MycAssay Aspergillus kit according to the manufacturer's instructions (Mycognostica, Manchester, United Kingdom). 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 4-point standard curve of $C_T$ values obtained with $10^3$ to $10^6$ *Aspergillus* CFU/ml. The area under the GI-versus-time curve was then correlated with the area under the PCR CE-versus-time curve by linear regression analysis.

**Pharmacodynamic analysis.** The GIs in the IC inoculated with $10^5$ CFU/ml *A. fumigatus*, *A. flavus*, or *A. terreus* were measured at regular time intervals up to 72 h. The pharmacodynamic data were subjected to nonlinear regression analysis based on the $E_{max}$ model as described above. The goodness of fit of the $E_{max}$ model was assessed by $R^2$ analysis of the residuals and visual inspection of the curves. The parameters $T_{50}$ and $E_{max}$ of GI-versus-time curves in the presence of drugs ($T_{50,D}$ and $E_{max,D}$) were compared with the corresponding parameters of drug-free growth controls ($T_{50,GC}$ and $E_{max,GC}$). The percent reduction was calculated as $100\% \times (E_{max,D} - E_{max,GC})/E_{max,GC}$, and the difference, $T_{50,GC} - T_{50,D}$, was calculated for each drug and species.

All of the experiments were repeated at least twice. All statistical analysis was performed with the software Prism 5.01 (GraphPad Inc., La Jolla, CA).

## RESULTS

**Validation of drug concentration determination by the bioassays.** The standard curves of inhibition zone diameters versus drug concentrations are shown in Fig. 2. The drug concentrations determined with the bioassay ranged from 0.5 mg/liter to 16 mg/liter. The diameter of the inhibition zone correlated linearly with the $\log_{10}$ drug concentration ($r^2$, >0.77). The intra- and interexperimental coefficients of variation ranged from 5% to 15%, with an average of 8%.

**Validation of fungal growth determination by measuring galactomannan production.** The production of galactomannan by *A. fumigatus* followed a sigmoid curve described very well by the $E_{max}$ model ($r^2$, >0.98) (Fig. 3). The parameter $T_{50}$ was inversely associated with the initial concentration of conidia ($r^2$, 0.98; slope, $-4.4 \pm 0.4$) and was therefore considered valid for quantifying fungal growth. Real-time PCR of *Aspergillus* DNA confirmed that the total increase in PCR CEs was associated with the total increase in galactomannan over time as assessed with the areas under the corresponding curves ($r^2$, 0.98) (Fig. 4). Although we did not assess the validity of using galactomannan to estimate the fungal burdens of other species/strains, any species/strain-dependent difference in galactomannan production will affect both drug-treated and drug-free control samples, hence without altering the net effect.

**Pharmacokinetic analysis.** The drug concentration-time pro-

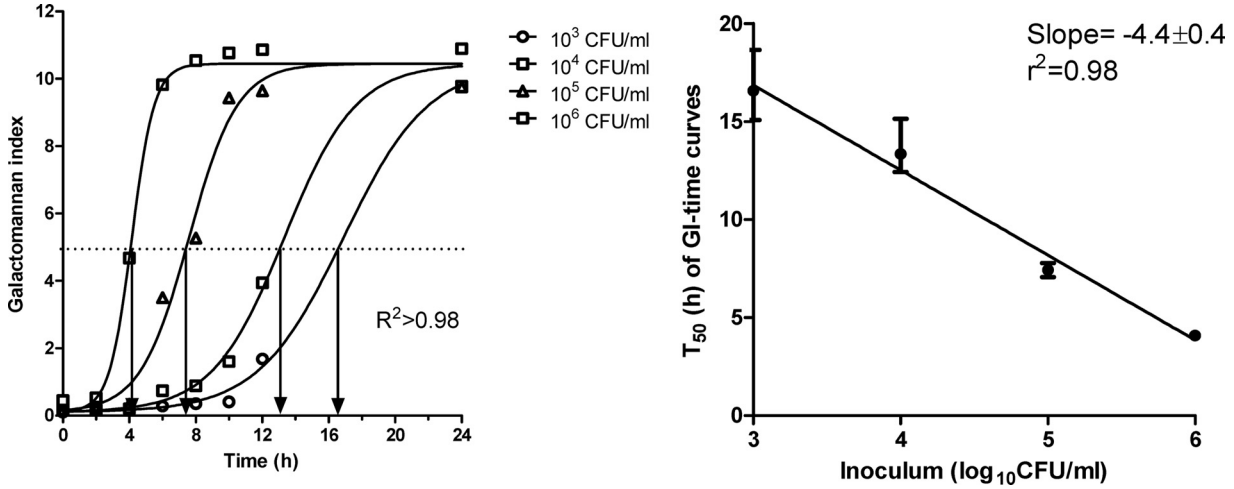


**FIG 3** Kinetics of galactomannan production by increasing inocula of *A. fumigatus* (left graph) and correlation of the $T_{50}$ parameter of the $E_{max}$ model with the initial inoculum (right graph). The arrows on the left graph show the $T_{50}$ parameter (the time corresponding to 50% of $E_{max}$) for each curve. Error bars represent 95% confidence intervals.

files of the three antifungal drugs in both the EC and the IC corresponded to those previously detected in human plasma after single-dose administration of 4 mg/kg voriconazole, 1 mg/kg caspofungin, and 1 mg/kg amphotericin B (2, 28, 34) (Fig. 5). The intra- and interexperimental variations in drug concentrations were <10% overall and <5% within the IC.

In particular, the $t_{1/2}$ of voriconazole in the *in vitro* model was 5.9 h with a maximum concentration of 3.7 mg/liter (Table 1). The corresponding values for caspofungin were 14 h and 9.3 mg/liter. These results are comparable to those for human plasma pharmacokinetic parameters, namely, 6.5 h and 3.62 mg/liter for voriconazole (2, 28, 34) and 12.2 h and 10.0 mg/liter for caspofungin (2, 28, 34). For amphotericin B, the maximum concentration (2.6 mg/liter) was similar to that in human plasma (2.83 mg/liter) whereas the $t_{1/2}$ was shorter than the average value (11 h and 19.65 h, respectively) (2, 28, 34). For all antifungal drugs, the $AUC_{0\text{-}24}$ values in the *in vitro* model were similar to the corresponding measurements in humans (Table 1).

**Pharmacodynamic analysis.** The pharmacodynamic data from the simulated doses of antifungal agents against the three isolates are shown in Fig. 6. The $E_{max}$ model described the data well, as demonstrated by an $R^2$ value of >0.86 and the normal distribution of residuals around 0. After 72 h of incubation and according to the $E_{max}$ parameter, amphotericin B completely inhibited the growth of *A. fumigatus* but not the growth of the other two strains (Table 2). According to the $T_{50}$ parameter, fungal growth was delayed for 7.53 h for *A. terreus*, 22.8 h for *A. flavus*, and >66.7 h for *A. fumigatus* (Table 2). Voriconazole inhibited the growth of *A. fumigatus* by 49.5% and that of *A. terreus* by 27.9% but had no effect on *A. flavus* growth. In contrast to *A. flavus*, for which voriconazole accelerated the production of galactomannan for 3.3 h, galactomannan production by *A. fumigatus* and *A. terreus* was delayed for 3.99 h and 5.37 h, respectively. Caspofungin did not have any effect on galactomannan production by *A. fumigatus* and *A. flavus*; whereas a delay of 2.8 h in galactomannan production was observed for *A. terreus*. When

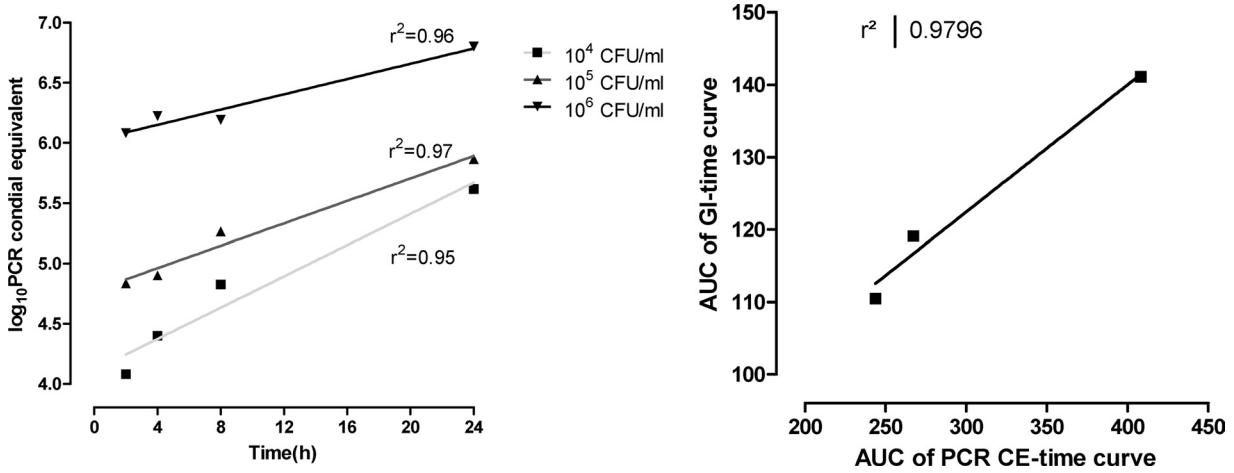


**FIG 4** Kinetics of real-time PCR CEs for three increasing inocula of *A. fumigatus* (left graph) and correlation between the area under the PCR CE-versus-time curve and the area under the GI-versus-time curve as in Fig. 2 (left graph). Results from experiments with a starting inoculum of $10^3$ CFU/ml were excluded from the analysis because the PCR signal was very close to the lower limit of detection, resulting in highly variable results.

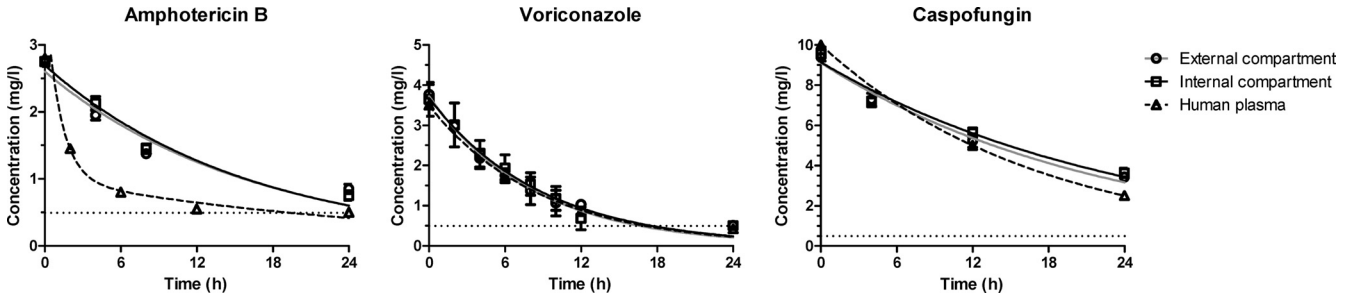


**FIG 5** Pharmacokinetic analysis of simulated doses of 1 mg/kg amphotericin B, 1 mg/kg caspofungin, and 4 mg/kg voriconazole in the new *in vitro* PK/PD model and comparison to results previously obtained by testing human plasma (2, 28, 34). Data represent drug levels in the EC (circles), IC (squares), and human plasma (triangles), and lines represent the regression lines obtained with the one-compartment pharmacokinetic intravenous model. Error bars represent standard deviations. The horizontal dotted lines represent the lower detection limits of the bioassays.

*Aspergillus* DNA was measured by real-time PCR, 2.2-, 0.9-, and 1.6-log reductions in the CEs of *A. fumigatus*, *A. flavus*, and *A. terreus*, respectively, were observed after 6 h of incubation in the *in vitro* PK/PD model in the presence of caspofungin compared to those of the drug-free controls (data not shown).

## DISCUSSION

Here we report for the first time, to our knowledge, a new *in vitro* pharmacokinetic model for *Aspergillus* species that simulates the human plasma pharmacokinetics of three major antimold agents (amphotericin B, caspofungin, and voriconazole) after the intravenous administration of standard doses (1 mg/kg, 1 mg/kg, and 4 mg/kg, respectively). For the pharmacodynamic analysis of these regimens, galactomannan production was determined as a surrogate marker of fungal growth. Three *Aspergillus* strains representing the most clinically significant species (*A. fumigatus*, *A. flavus*, and *A. terreus*) were tested by this model. The isolate of each species was deliberately selected for identical MICs of a given antifungal drug. Thus, the MICs were the same for amphotericin B (1 mg/liter) and voriconazole (0.5 mg/liter) and the MECs were also the same (0.5 mg/liter) in order to elucidate the species-specific *in vitro* PK/PD effects of a given antifungal drug that were not necessarily reflected by the MIC.

**TABLE 1** Pharmacokinetic parameters of amphotericin B, caspofungin, and voriconazole in humans and in the *in vitro* PK/PD model

| Drug (simulated dose [mg/kg]) and pharmacokinetic parameter[a] | Avg value in human plasma[b] | Mean value in IC[c] ± SEM |
|---|---|---|
| Amphotericin B (1) | | |
| $C_{max}$ (mg/liter) | 2.83 | 2.6 ± 0.1 |
| $t_{1/2}$ (h) | 19.65 | 11 ± 1.5 |
| $AUC_{0\text{-}24}$ (mg · h/liter) | 28.98 | 34.52 |
| Voriconazole (4) | | |
| $C_{max}$ (mg/liter) | 3.62 | 3.7 ± 0.17 |
| $t_{1/2}$ (h) | 6.5 | 5.9 ± 0.6 |
| $AUC_{0\text{-}24}$ (mg · h/liter) | 22.7 | 30.37 |
| Caspofungin (1) | | |
| $C_{max}$ (mg/liter) | 10 | 9.3 ± 0.25 |
| $t_{1/2}$ (h) | 12.2 | 14 ± 1.25 |
| $AUC_{0\text{-}24}$ (mg · h/liter) | 97.20 | 120.31 |

[a] $C_{max}$, maximum drug concentration in human plasma.
[b] Data derived from previous clinical studies (2, 28, 34).
[c] Obtained with the *in vitro* PK/PD model.

The *in vitro* pharmacokinetic parameters were similar to those observed in humans for all the three drugs except amphotericin B, for which the $t_{1/2}$ in the *in vitro* model was around 11 h, as opposed to 19 h, which represents the mean residence time of amphotericin B in plasma after noncompartmental analysis. Compartmental analysis showed a rapid distribution A phase with a $t_{1/2}$ of $<1$ h observed at 0 to 6 h and a slower elimination B phase with a $t_{1/2}$ of 15 h observed 6 to 24 h after intravenous administration (2, 3). This divergence has also been observed in previous *in vitro* models developed for the study of *Candida* species and has been attributed to amphotericin B degradation during incubation at 37°C (13). This difference is unlikely to affect the drug's pharmacodynamics, as our *in vitro* PK/PD model accurately captures the peak concentration of amphotericin B, which is the critical pharmacodynamic variable of this polyene antifungal agent.

Amphotericin B was for decades the treatment of choice for aspergillosis. Clinical and experimental data indicated differences in efficacy against infections caused by various *Aspergillus* species (6). Lack of clinical efficacy, however, was not associated with significantly increased MICs (12, 18, 21), which are similar for all three species, with slightly higher MICs of amphotericin B for *A. terreus* (20). In the present study, the *Aspergillus* isolates exhibited the same MIC (1 mg/liter), classified as susceptible (4), suggesting similar efficacies of amphotericin B. However, the new *in vitro* model revealed differences in the efficacy of amphotericin B against the three *Aspergillus* species, 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 infection cause by *A. fumigatus* than against infection with *A. flavus* and less effective against infection with *A. terreus* (23, 36). It seems that the new *in vitro* model described here may better characterize the pharmacodynamic characteristics of amphotericin B for the major *Aspergillus* species tested than conventional *in vitro* susceptibility systems can.

Currently, voriconazole is the drug of choice for the treatment of aspergillosis (10). In general, the MICs of voriconazole for most of the isolates of *A. fumigatus*, *A. flavus*, and *A. terreus* are similar (20). However, there are significant differences in the *in vivo* activities of voriconazole against infections caused by these three *Aspergillus* species (32, 37). Although each isolate of the three *Aspergillus* species used in the present study had the same MIC

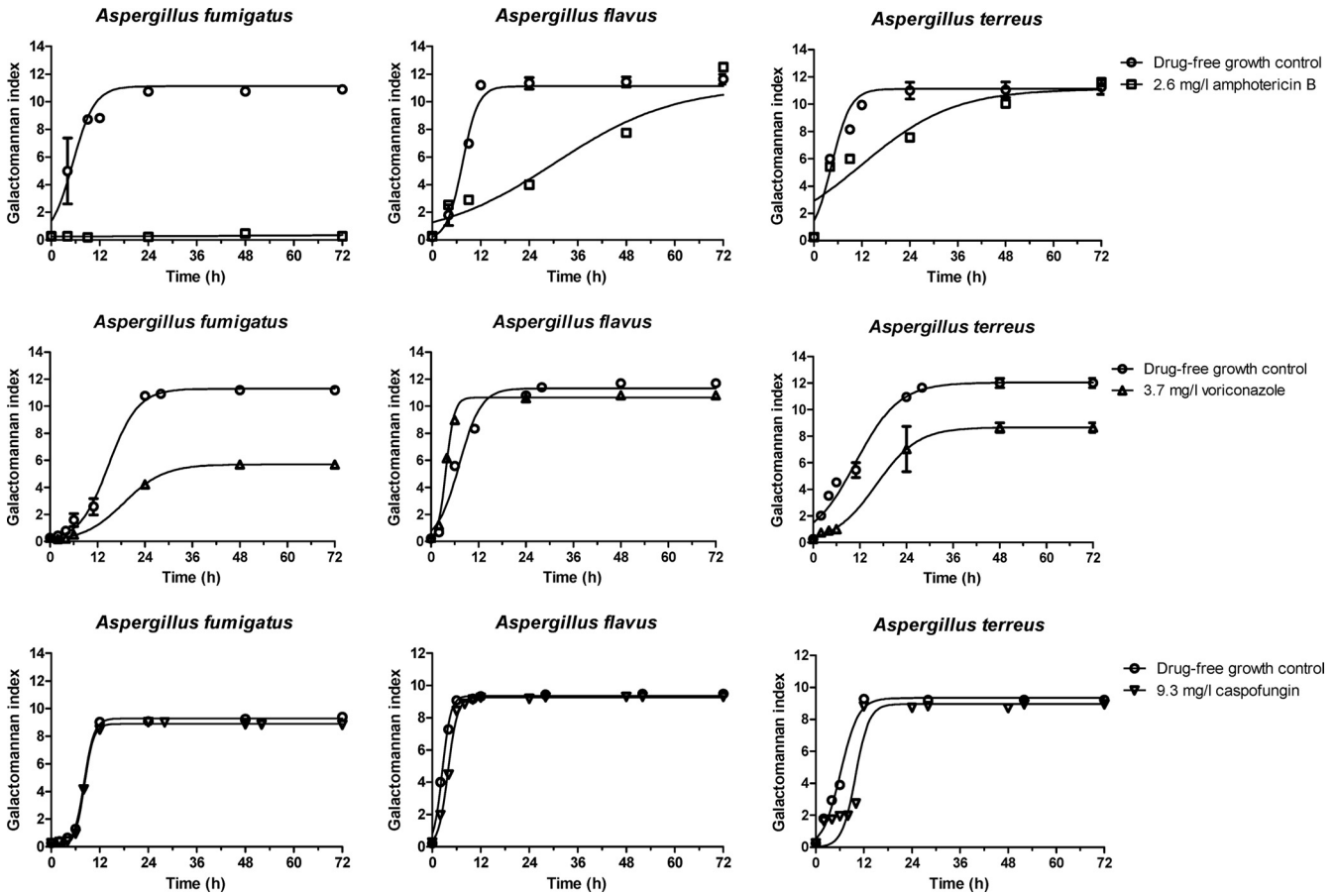


**FIG 6** Pharmacodynamic data analysis according to the *in vitro* PK/PD model. Simulated human dosing with 1 mg/kg amphotericin B, 4 mg/kg voriconazole, or 1 mg/kg caspofungin was used against *A. fumigatus*, *A. flavus*, and *A. terreus* clinical isolates. The circles and squares represent galactomannan indices obtained in experiments without and with the drugs, respectively, while the lines represent the regression lines obtained with the $E_{max}$ model.

(0.5 mg/liter), the new *in vitro* PK/PD model revealed significant pharmacodynamic differences, with the greatest efficacy of voriconazole found against *A. fumigatus* and less against *A. terreus*; whereas no activity against *A. flavus* was detected. Voriconazole at a dose of 10 mg/kg, which results in a drug exposure in mice similar to that achieved with standard doses in humans, is active against *A. fumigatus* (22, 32) but not against *A. flavus* (37) infections in murine models of invasive aspergillosis. In clinical studies, the survival rate of patients with invasive aspergillosis caused by *A. terreus* is generally lower than that in infections with *A. fumigatus* after treatment with voriconazole (10, 31). Of note, voriconazole showed better efficacy than other antifungals against *A. terreus* infections (31), as in our study. These *in vivo* and clinical data suggest that voriconazole may be more active against *A. fumigatus* than against *A. terreus* and may have little activity against *A. flavus*, in agreement with the findings of the new *in vitro* model. Thus, the new *in vitro* PK/PD model revealed clinical and *in vivo* relevant pharmacodynamic differences in voriconazole efficacy against the three *Aspergillus* spp. tested.

Caspofungin is used as salvage therapy against invasive aspergillosis, although recent studies suggest its effectiveness as first-line therapy (11). In the new *in vitro* model, caspofungin did not demonstrate any effect on the growth of an *Aspergillus* species other than *A. terreus*, in which galactomannan production was simply delayed for 2.83 h. Caspofungin inhibits 1,3-$\beta$-D-glucan synthesis, which results in distortion of the cell wall and suppression of growth. Galactomannan may be released during the process of cell wall disruption. Consequently, galactomannan levels may remain high despite effective antifungal activity. Thus, for pharmacodynamic study of the actions of caspofungin and other echinocandins, measurement of galactomannan production appears not to be informative either *in vitro* and *in vivo* (26). Another biomarker is needed to monitor the pharmacodynamics of echinocandins. An alternative endpoint for determining the activity of antifungal drugs, and particularly of echinocandins, may be PCR CEs.

In conclusion, in the present study, we developed and for the first time reported an *in vitro* PK/PD model which simulates the pharmacokinetics of three major antifungal drugs in humans and assesses their efficacy against three clinically significant *Aspergillus* species. The species-dependent pharmacodynamic differences revealed with the *in vitro* PK/PD model are also reflected by the different epidemiological cutoff values found for these *Aspergillus* species (7), in line with the species-specific breakpoints for *Candida* spp. (27, 29), and may be attributed to different genetic background (33), particularly for genes involved in growth and drug transport (8). Because total drug concentrations were simulated in the *in vitro* PK/PD model, the drug that is available for microbio-

**TABLE 2** Pharmacodynamic parameters of simulated doses of amphotericin B, voriconazole, and caspofungin against *A. fumigatus*, *A. flavus*, and *A. terreus* isolates in the *in vitro* PK/PD model

| Drug (simulated dose [mg/kg]) and species | Mean $E_{max}$[a] (GI) ± SD | | | Mean $T_{50}$[b] (h) ± SD | | |
|---|---|---|---|---|---|---|
| | With drug | Without drug | % Change | With drug | Without drug | Difference |
| Amphotericin B (1) | | | | | | |
| *A. fumigatus* | 10.22 ± 0.65 | 0 | −100[c] | 5.3 ± 0.7 | >72 | >−66.7[c] |
| *A. flavus* | 11.54 ± 0.21 | 11.12 ± 0.26 | −3.6 | 7.5 ± 0.67 | 30.31 ± 4.24 | −22.8[c] |
| *A. terreus* | 10.72 ± 0.36 | 10.96 ± 1.73 | 2.2 | 4.45 ± 0.61 | 11.98 ± 2.84 | −7.53[c] |
| Voriconazole (4) | | | | | | |
| *A. fumigatus* | 11.29 ± 0.18 | 5.70 ± 0.03 | −49.5[c] | 14.81 ± 0.61 | 18.80 ± 0.25 | −3.99[c] |
| *A. flavus* | 11.35 ± 0.43 | 10.63 ± 0.14 | −6.3 | 7.13 ± 0.76 | 3.82 ± 0.09 | 3.31[c] |
| *A. terreus* | 12.02 ± 0.34 | 8.66 ± 0.29 | −27.9[c] | 10.92 ± 0.93 | 16.29 ± 1.33 | −5.37[c] |
| Caspofungin (1) | | | | | | |
| *A. fumigatus* | 9.24 ± 0.08 | 8.97 ± 0.08 | −2.9 | 8.28 ± 0.30 | 8.22 ± 0.07 | −0.02 |
| *A. flavus* | 9.38 ± 0.06 | 9.21 ± 0.04 | −1.8 | 3.26 ± 2.1 | 4.41 ± 0.45 | 1.15 |
| *A. terreus* | 9.27 ± 0.48 | 8.83 ± 0.06 | −4.7 | 7.68 ± 0.49 | 10.48 ± 0.52 | 2.8[c] |

[a] $E_{max}$, maximum GI.
[b] $T_{50}$, time required to obtain 50% of $E_{max}$.
[c] Statistically significantly different ($P < 0.05$).

logic activity may be overestimated due to protein binding *in vivo*. Furthermore, this model should be validated with results of *in vivo* antifungal efficacy from animal experiments. This model can be adapted to incorporate serum, neutrophils, and other host factors that may affect antifungal pharmacodynamics. It can also be used for exploring more effective dosing regimens with dose escalation and fractionation studies, for PK/PD modeling and for studying the activity of drug combinations in order to improve the treatment and prognosis of *Aspergillus* and even other mold infections.

## ACKNOWLEDGMENTS

This study was supported by Marie Curie Reintegration Grant MIRG-CT-2007-208796 from the European Commission, Bodosakis Foundation, FLOGA, and the University of Athens SARG 70/3/6915 for infrastructural subsidy.

## REFERENCES

1. **Andes D.** 2006. Pharmacokinetics and pharmacodynamics of antifungals. Infect. Dis. Clin. North Am. **20**:679–697.
2. **Ayestarán A, et al.** 1996. Pharmacokinetics of conventional formulation versus fat emulsion formulation of amphotericin B in a group of patients with neutropenia. Antimicrob. Agents Chemother. **40**:609–612.
3. **Bekersky I, et al.** 2002. Pharmacokinetics, excretion, and mass balance of liposomal amphotericin B (AmBisome) and amphotericin B deoxycholate in humans. Antimicrob. Agents Chemother. **46**:828–833.
4. **Cantón E, Espinel-Ingroff A, Peman J.** 2009. Trends in antifungal susceptibility testing using CLSI reference and commercial methods. Expert Rev. Anti Infect. Ther. **7**:107–119.
5. **CLSI.** 2008. Reference method for broth dilution antifungal susceptibility testing of filamentous fungi; approved standard. Second edition. CLSI document M38-A2. Clinical and Laboratory Standards Institute, Wayne, PA.
6. **Denning DW, et al.** 1998. An EORTC multicentre prospective survey of invasive aspergillosis in haematological patients: diagnosis and therapeutic outcome. J. Infect. **37**:173–180.
7. **Espinel-Ingroff A, et al.** 2010. Wild-type MIC distributions and epidemiological cutoff values for the triazoles and six *Aspergillus* spp. for the CLSI broth microdilution method (M38-A2 document). J. Clin. Microbiol. **48**:3251–3257.
8. **Fedorova ND, et al.** 2008. Genomic islands in the pathogenic filamentous fungus Aspergillus fumigatus. PLoS Genet. **4**:e1000046.
9. **Groll AH, Piscitelli SC, Walsh TJ.** 1998. Clinical pharmacology of systemic antifungal agents: a comprehensive review of agents in clinical use, current investigational compounds, and putative targets for antifungal drug development. Adv. Pharmacol. **44**:343–500.
10. **Herbrecht R, et al.** 2002. Voriconazole versus amphotericin B for primary therapy of invasive aspergillosis. N. Engl. J. Med. **347**:408–415.
11. **Herbrecht R, et al.** 2010. Caspofungin first-line therapy for invasive aspergillosis in allogeneic hematopoietic stem cell transplant patients: an European Organisation for Research and Treatment of Cancer study. Bone Marrow Transplant. **45**:1227–1233.
12. **Johnson EM, et al.** 2000. Lack of correlation of in vitro amphotericin B susceptibility testing with outcome in a murine model of Aspergillus infection. J. Antimicrob. Chemother. **45**:85–93.
13. **Lewis RE, Kontoyiannis DP, Darouiche RO, Raad II, Prince RA.** 2002. Antifungal activity of amphotericin B, fluconazole, and voriconazole in an in vitro model of *Candida* catheter-related bloodstream infection. Antimicrob. Agents Chemother. **46**:3499–3505.
14. **Lewis RE, Lund BC, Klepser ME, Ernst EJ, Pfaller MA.** 1998. Assessment of antifungal activities of fluconazole and amphotericin B administered alone and in combination against *Candida albicans* by using a dynamic in vitro mycotic infection model. Antimicrob. Agents Chemother. **42**:1382–1386.
15. **Lewis RE, Wiederhold NP, Prince RA, Kontoyiannis DP.** 2006. In vitro pharmacodynamics of rapid versus continuous infusion of amphotericin B deoxycholate against Candida species in the presence of human serum albumin. J. Antimicrob. Chemother. **57**:288–293.
16. **Li RC, Zhu ZY.** 1997. In vitro models for prediction of antimicrobial activity: a pharmacokinetic and pharmacodynamic perspective. J. Chemother. **9**(Suppl. 1):55–63.
17. **Lignell A, Johansson A, Lowdin E, Cars O, Sjolin J.** 2007. A new in-vitro kinetic model to study the pharmacodynamics of antifungal agents: inhibition of the fungicidal activity of amphotericin B against Candida albicans by voriconazole. Clin. Microbiol. Infect. **13**:613–619.
18. **Lionakis MS, Lewis RE, Chamilos G, Kontoyiannis DP.** 2005. Aspergillus susceptibility testing in patients with cancer and invasive aspergillosis: difficulties in establishing correlation between in vitro susceptibility data and the outcome of initial amphotericin B therapy. Pharmacotherapy **25**:1174–1180.
19. **Marr KA, Patterson T, Denning D.** 2002. Aspergillosis. Pathogenesis, clinical manifestations, and therapy. Infect. Dis. Clin. North Am. **16**:875–894.
20. **Meletiadis J, et al.** 2007. Differential fungicidal activities of amphotericin B and voriconazole against *Aspergillus* species determined by microbroth methodology. Antimicrob. Agents Chemother. **51**:3329–3337.
21. **Mosquera J, et al.** 2001. Susceptibility testing of *Aspergillus flavus*: inoculum dependence with itraconazole and lack of correlation between susceptibility to amphotericin B in vitro and outcome in vivo. Antimicrob. Agents Chemother. **45**:1456–1462.

22. **Murphy M, Bernard EM, Ishimaru T, Armstrong D.** 1997. Activity of voriconazole (UK-109,496) against clinical isolates of *Aspergillus* species and its effectiveness in an experimental model of invasive pulmonary aspergillosis. Antimicrob. Agents Chemother. **41**:696–698.
23. **Odds FC, et al.** 1998. Evaluation of possible correlations between antifungal susceptibilities of filamentous fungi in vitro and antifungal treatment outcomes in animal infection models. Antimicrob. Agents Chemother. **42**:282–288.
24. **Patterson TF, et al.** 2000. Invasive aspergillosis. Disease spectrum, treatment practices, and outcomes. Medicine (Baltimore) **79**:250–260.
25. **Perea S, et al.** 2000. Comparison of high-performance liquid chromatographic and microbiological methods for determination of voriconazole levels in plasma. Antimicrob. Agents Chemother. **44**:1209–1213.
26. **Petraitiene R, et al.** 2002. Antifungal efficacy of caspofungin (MK-0991) in experimental pulmonary aspergillosis in persistently neutropenic rabbits: pharmacokinetics, drug disposition, and relationship to galactomannan antigenemia. Antimicrob. Agents Chemother. **46**:12–23.
27. **Pfaller MA, Andes D, Diekema DJ, Espinel-Ingroff A, Sheehan D.** 2010. Wild-type MIC distributions, epidemiological cutoff values and species-specific clinical breakpoints for fluconazole and Candida: time for harmonization of CLSI and EUCAST broth microdilution methods. Drug Resist. Updat. **13**:180–195.
28. **Purkins L, et al.** 2003. The pharmacokinetics and safety of intravenous voriconazole—a novel wide-spectrum antifungal agent. Br. J. Clin. Pharmacol. **56**(Suppl. 1):2–9.
29. **Rodríguez-Tudela JL, Arendrup MC, Cuenca-Estrella M, Donnelly JP, Lass-Florl C.** 2010. EUCAST breakpoints for antifungals. Drug News Perspect. **23**:93–97.
30. **Shadomy S, McCay JA, Schwartz SI.** 1969. Bioassay for hamycin and amphotericin B in serum and other biological fluids. Appl. Microbiol. **17**:497–503.
31. **Steinbach WJ, et al.** 2004. Infections due to *Aspergillus terreus*: a multicenter retrospective analysis of 83 cases. Clin. Infect. Dis. **39**:192–198.
32. **Takemoto K, et al.** 2009. Comparative study on the efficacy of liposomal amphotericin B and voriconazole in a murine pulmonary aspergillosis model. Chemotherapy **55**:105–113.
33. **Tekaia F, Latge JP.** 2005. Aspergillus fumigatus: saprophyte or pathogen? Curr. Opin. Microbiol. **8**:385–392.
34. **Walsh TJ, et al.** 2005. Pharmacokinetics, safety, and tolerability of caspofungin in children and adolescents. Antimicrob. Agents Chemother. **49**:4536–4545.
35. **Walsh TJ, et al.** 2008. Treatment of aspergillosis: clinical practice guidelines of the Infectious Diseases Society of America. Clin. Infect. Dis. **46**:327–360.
36. **Walsh TJ, et al.** 2003. Experimental pulmonary aspergillosis due to *Aspergillus terreus*: pathogenesis and treatment of an emerging fungal pathogen resistant to amphotericin B. J. Infect. Dis. **188**:305–319.
37. **Warn PA, et al.** 2006. Comparative in vivo activity of BAL4815, the active component of the prodrug BAL8557, in a neutropenic murine model of disseminated *Aspergillus flavus*. J. Antimicrob. Chemother. **58**:1198–1207.