In Vitro Pharmacodynamics of Amphotericin B, Itraconazole, and Voriconazole against *Aspergillus*, *Fusarium*, and *Scedosporium* spp.

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We compared the *in vitro* pharmacodynamics of amphotericin B, itraconazole, and voriconazole against *Aspergillus*, *Fusarium*, and *Scedosporium* species with a combination of two non-culture-based techniques: the tetrazolium salt 2,3-bis-(2-methoxy-4-nitro-5-[(sulfenylamino)carbonyl]-2H-tetrazolium-hydroxide) (XTT) colorimetric reduction assay, and fluorescent microscopy with the cellular morbidity dye bis-(1,3-dibutylbarbituric acid) trimethine oxonol (DiBAC) to directly visualize hyphal damage. Amphotericin B exhibited species-specific concentration-dependent activity, with 50% effective concentrations (EC_{50} s) ranging from 0.10 to 0.12 mg/ml for *A. fumigatus*, 0.36 to 0.53 mg/ml for *A. terreus*, 0.27 to ≥ 32 mg/ml for *F. solani*, 0.41 to 0.55 mg/ml for *F. oxysporum*, and 0.97 and 0.65 mg/ml for *S. apiospermum* and *S. prolificans*, respectively. Similarly, itraconazole inhibited the growth of *A. fumigatus* and *A. terreus* isolates with MICs of <1 mg/ml (EC_{50} 0.03 to 0.85 mg/ml) and *S. apiospermum*, but was not active against *Fusarium* species or *S. prolificans*. Voriconazole effectively inhibited the growth of *Aspergillus*, *Fusarium*, and *S. apiospermum* (EC_{50} 0.10 to 3.3 mg/ml) but had minimal activity against a multidrug-resistant isolate of *F. solani* or *S. prolificans*. Hyphal damage visualized by DiBAC staining was observed more frequently with voriconazole and amphotericin B versus itraconazole. These data highlight the species-specific differences in antifungal pharmacodynamics between mold-active agents that could be relevant for the development of *in vitro* susceptibility breakpoints and antifungal dosing *in vivo*.

Infections caused by the opportunistic mold genera *Aspergillus*, *Fusarium*, and *Scedosporium* have increased over the last decade and are associated with significant morbidity and mortality in severely immunocompromised patients (10, 12). *In vitro* studies have revealed that some *Aspergillus* species, particularly *A. terreus*, and most *Fusarium* and *Scedosporium* isolates are only moderately susceptible or resistant to amphotericin B (18). Similarly, itraconazole appears to have negligible activity against *Fusarium* and *Scedosporium* species (18), which have become increasingly common causes of breakthrough infections in immunosuppressed patients (7). More recently, voriconazole has been shown to be effective as primary therapy in the treatment of invasive aspergillosis and is an effective salvage therapy for refractory infections caused by *Fusarium* species and *Scedosporium apiospermum* (17). However, no comparative studies have directly compared the pharmacodynamics of amphotericin B, itraconazole, and voriconazole for *Aspergillus*, *Fusarium*, and *Scedosporium* species. Comparative pharmacodynamic data for these molds, especially *Fusarium* and *Scedosporium*, could be informative in the development of susceptibility breakpoints as well as dosing regimen design for infections not evaluated in phase I and II clinical trials (19).

One factor that has hindered the study of antifungal pharmacodynamics for opportunistic molds both *in vitro* and *in vivo* is the lack of standardized, reliable, and sensitive techniques

for quantifying viable mycelium. Methods developed for unicellular organisms (e.g., time-kill curves and quantitative organ cultures) that rely on CFU enumeration from agar medium are not well suited for multicellular filamentous fungi that may only grow as a single CFU when plated on agar (4). Moreover, in studies that have attempted to apply traditional time-kill methods for studies of antifungal activity against *Aspergillus* spp., CFU counts in control tubes decreased despite obvious increases in hyphal mass and the slope of antifungal “killing” in control tubes was often indistinguishable from that of drug-exposed isolates (11).

Recently, colorimetric assays and quantitative real-time PCR have been proposed as alternatives to CFU-based methods as *in vitro* and *in vivo* endpoints, respectively, for the study of drug activity against filamentous fungi (4, 13). *In vitro*, the reduction of the tetrazolium salt 2,3-bis-(2-methoxy-4-nitro-5-[(sulfenylamino)carbonyl]-2H-tetrazolium-hydroxide) (XTT) by viable hyphae can serve as an indicator of hyphal viability and has been shown to reliably and accurately quantify fungal burden following 24 h of incubation with as few as 10^2 conidia (13). Therefore, we used the XTT reduction assay combined with fluorescent microscopy with the cellular morbidity dye bis-(1,3-dibutylbarbituric acid) trimethine oxonol (DiBAC) as an early marker of hyphal damage to characterize the *in vitro* pharmacodynamics of amphotericin B, itraconazole, and voriconazole against *Aspergillus*, *Fusarium*, and *Scedosporium* species with various susceptibilities to amphotericin B and itraconazole. With this combined approach, we were able to characterize the concentration-effect relationships of all three

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antifungals against a variety of pathogenic molds and confirm (qualitatively) differences in drug-induced hyphal damage between the three antifungals.

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MATERIALS AND METHODS

Isolates and inoculum preparation. Eleven isolates with various susceptibilities to amphotericin B and itraconazole were selected for testing from the laboratory collection at the Mycology Research Laboratories at the University of Texas M. D. Anderson Cancer Center, Houston. All isolates were subcultured twice on potato dextrose agar slants (Remel, Lenexa, Kans.) prior to testing. A standardized inoculum of conidia for each isolate was prepared by flooding 5- to 7-day-old cultures on potato dextrose agar slants with 5 ml of 0.85% sterile saline and 0.2% Tween 80 followed by 15 s of vortexing. The resultant suspension was then filtered twice through sterile syringes packed with glass wool to remove hyphal fragments. The conidial suspension was adjusted with a hemacytometer to a concentration of 1×10^6 to 5×10^6 in sterile water (16).

Culture medium. RPMI 1640 culture medium (Sigma) buffered with 0.165 M MOPS (3-[N-morpholino]propanesulfonic acid) plus 2% glucose adjusted to a pH of 7.0 served as the growth medium for all experiments.

Drug stock solutions. Drug stock solutions (1,280 mg/ml) were prepared for each antifungal in dimethyl sulfoxide (voriconazole and itraconazole) or water (amphotericin B deoxycholate) from pure powders (voriconazole from Pfizer, Inc, Sandwich, United Kingdom; itraconazole from Janssen Inc., Titusville, N.J.; amphotericin B deoxycholate from Sigma). Stock solutions were then prepared in RPMI culture medium and serially diluted (64 to 0.0625 $\mu\text{g/ml}$) for preparation of microtiter trays.

Antifungal susceptibility testing. The MIC of each antifungal was determined in triplicate with standardized methods (NCCLS M38-A) (16). Briefly, conidial suspensions were diluted 1:50 in RPMI growth medium and dispensed (100 μl) into thawed microtiter trays containing serial twofold dilutions of antifungals. The trays were then incubated for 48 h at 37°C, and MICs were read at 24 and 48 h as the lowest drug concentrations that showed complete growth inhibition (100% inhibition). *Candida parapsilosis* ATCC 22019 served as a quality control isolate for each experimental run. The in vitro fungicidal activity (minimum fungicidal activity [MFC]) of each antifungal was measured for each isolate with previously reported methods (6). After 48 h of incubation, 20 μl was subcultured from each well that showed complete inhibition (100% or an optically clear well), from the last positive well (growth similar to that for the growth control well), and from the growth control (drug-free medium) onto potato dextrose agar plates (Remel, Lenexa, Kans.). The plates were incubated in a humid chamber at 35°C until growth was seen in the growth control subculture (usually before 48 h). The MFC was defined as the lowest drug concentration that showed either no growth or fewer than three colonies to achieve approximately 99 to 99.5% killing activity (6).

XTT reduction assay. The XTT reduction assay was performed with methods developed by Meletiadis et al. (13). The assay relies on reduction of the tetrazolium salt XTT by intact cells with active biological redox capabilities to formazan derivatives, which can be measured colorimetrically (20). The relationship of formazan production versus fungal inoculum was determined by incubating standardized conidial suspensions of each isolate (10^2 to 10^5 conidia/ml) in RPMI growth medium for 24 h at 35°C. Additional wells containing medium alone were included in each plate to correct for background absorbance and to verify growth medium sterility. After 22 h of incubation, the trays were removed from the incubator and 50 ml of XTT solution (1 mg/ml) prepared in sterile water containing 125 mM menadione (Sigma) was added to each well. After 2 additional hours of incubation, formazan absorbance in each well was read at 492 nm with a microplate spectrophotometer (Powerwave X Select, Biotech Instruments, Winooski, Vt.). Absorbance readings were standardized in relation to unconverted XTT in the medium control wells and plate absorbance (692 nm) to determine the optical density at 492 nm. All experiments were performed in triplicate.

Pharmacodynamic studies were performed by inoculating 100 μl of a $1:5$ dilution of the standardized conidial suspension (1×10^6 to 2×10^6 conidia/ml) into the wells of a flat-bottomed 96-well microtiter tray containing serial twofold dilutions of antifungals in RPMI growth medium (final concentration, 0.0625 to 32 $\mu\text{g/ml}$) plus a control (no drug). The trays were then incubated for 24 h at 35°C, and formazan absorbance was assayed as described previously. All experiments were performed in triplicate.

Fluorescent morbidity staining. Fluorescent morbidity staining and microscopy were performed with a modification of the methods reported by Bowman et al. (5). Briefly, isolates were grown in RPMI medium for 18 h prior to the addition of amphotericin B, itraconazole, or voriconazole to the culture medium to a final concentration of 2 $\mu\text{g/ml}$. After incubation of cells for an additional 6 h, tubes were centrifuged at $10,000 \times g$ for 5 min, culture medium was carefully removed with a sterile pipette, and fungi were washed twice in morpholinepropanesulfonic acid (MOPS, pH 7) buffer solution. DiBAC (Molecular Probes, Eugene, Oreg.) from a 1-mg/ml stock in 100% ethanol was then added to a final concentration of 2 mg/ml, and samples were incubated in the dark with shaking at room temperature for 1 h, washed twice again with MOPS, pH 7, and resuspended for photomicrography.

Photomicrographs of hyphae were taken with an Olympus BX-51 microscope equipped with Nomarski optics and epifluorescence (fluorescein isothiocyanate) filter. All pictures were taken at $400\times$ magnification with a computerized digital camera (Spot Diagnostics, Sterling Heights, Mich.). The exposure times and light balance of micrographs were controlled through computer software (Image Pro Plus 4.1, Media Cybernetics, Silver Spring, Md.).

Data analysis. Median MIC and MFC values were calculated from experiments performed in triplicate. Data from the XTT reduction assay were fitted to a four-parameter logistic model (Hill Equation) with computer curve-fitting software (Prism 4, Graphpad Software, Inc., San Diego, Calif.) to derive the 50% effective concentration (EC_{50}) and the steepness of the inhibitory dose-response curve (Hill slope) (15). The goodness of fit for each isolate and drug combination was assessed by R^2 and the standard error of the EC_{50} value. If the standard error of the EC_{50} was ≥ 0.5 log, data were refitted with a fixed value for the top plateau of the regression curve based on the formazan absorbance of the control wells. The EC_{50} and Hill slope data were not determined if minimal or no reduction in formazan absorbance was observed with increasing drug concentrations.

RESULTS

Antifungal susceptibility testing. Median MIC and MFC results are reported in Table 1. Amphotericin B MICs were relatively lower for *A. fumigatus* (MIC 0.25 $\mu\text{g/ml}$) than for *Aspergillus terreus*, *Fusarium solani* 001, *Fusarium oxysporum* 001 and 002, and *Scedosporium apiospermum*, all of which had MICs of ≥ 1 $\mu\text{g/ml}$. The MFCs for amphotericin B were 2 $\mu\text{g/ml}$ or greater for all isolates with the exception of *A. fumigatus* isolates 99-2075 and 583 (0.5 $\mu\text{g/ml}$). Similarly, higher MICs of itraconazole (≥ 2 $\mu\text{g/ml}$) were observed with *A. fumigatus* 583 and all *Fusarium* and *Scedosporium* test isolates. Itraconazole MFCs ranged between 8 and 32 $\mu\text{g/ml}$ for all test isolates. Median voriconazole MICs were 0.25 $\mu\text{g/ml}$, and MFCs ranged from 1 to 4 $\mu\text{g/ml}$ for all *Aspergillus* species tested. Voriconazole MICs and MFCs were several dilutions higher with *Fusarium* species (2 to 16 $\mu\text{g/ml}$ and ≥ 32 $\mu\text{g/ml}$, respectively) compared to *Aspergillus*. Voriconazole was considerably more active against *S. apiospermum* (MIC and MFC, 0.25 and 16 $\mu\text{g/ml}$, respectively) versus *S. prolificans* (16 and ≥ 32 $\mu\text{g/ml}$, respectively).

XTT reduction assay. A linear relationship between XTT reduction to formazan, the colorimetrically assayed product, and starting inoculum was observed for isolates tested between 10^2 and 10^5 conidia/ml (regression plots are presented in Fig. 1). Slopes of regression plots for all 11 isolates ranged from 0.45 to 0.63, with coefficients of determination (R^2) ranging from 0.78 to 0.94. Addition of amphotericin B, itraconazole, or voriconazole to culture medium containing susceptible molds reduced formazan absorbance from approximately 3 to ≤ 0.5 , which was roughly equivalent to a 2.5 log reduction in hyphal viability based on data generated from inoculum-XTT reduction studies (Fig. 1).

Pharmacodynamic results for all antifungal-isolate combinations are presented in Table 1, with concentration-effect plots

TABLE 1. MICs and MFCs^a

Isolate	Amphotericin B			Itraconazole			Voriconazole		
	MIC/MFC (μg/ml)	EC ₅₀ (μg/ml) ± 95% CI	Hill slope ± 95% CI	MIC/MFC (μg/ml)	EC ₅₀ (μg/ml) ± 95% CI	Hill slope ± 95% CI	MIC/MFC (μg/ml)	EC ₅₀ (μg/ml) ± 95% CI	Hill slope ± 95% CI
<i>A. fumigatus</i> 293	0.25/2	0.10 ± 0.06	-3.84 ± 2.10	0.25/2	0.30 ± 0.10	-2.40 ± 1.00	0.25/0.50	0.18 ± 0.06	-3.07 ± 1.10
<i>A. fumigatus</i> 99-2075	0.25/0.5	0.12 ± 0.08	-3.25 ± 1.20	0.5/16	0.85 ± 0.46	-0.56 ± 0.45	0.25/1	0.10 ± 0.02	-2.66 ± 0.60
<i>A. fumigatus</i> 583	0.25/0.5	0.10 ± 0.01	-4.27 ± 2.10	32/≥32	12.3 ± 2.00	-0.56 ± 0.31	0.25/2	0.37 ± 0.07	-1.20 ± 0.23
<i>A. terreus</i> 0925	1/2	0.53 ± 0.10	-5.33 ± 3.20	1/8	0.83 ± 0.12	-3.37 ± 1.82	0.25/4	0.68 ± 0.19	-2.15 ± 0.62
<i>A. terreus</i> 0932	1/4	0.36 ± 0.06	-2.65 ± 2.10	0.5/8	0.24 ± 0.05	-3.13 ± 2.00	0.25/4	0.10 ± 0.04	-1.43 ± 0.60
<i>F. solani</i> 001	16/≥32	NC ^b	NC	≥32/≥32	NC	NC	16/≥32	NC	NC
<i>F. solani</i> 002	1/2	0.27 ± 0.12	-1.62 ± 0.63	8/≥32	NC	NC	4/32	3.30 ± 1.30	-0.87 ± 0.50
<i>F. oxysporum</i> 001	4/8	0.55 ± 0.35	-0.86 ± 0.66	≥32/≥32	NC	NC	4/≥32	3.12 ± 1.70	-0.81 ± 0.67
<i>F. oxysporum</i> 002	2/4	0.41 ± 0.26	-0.80 ± 0.30	8/≥32	NC	NC	2/≥32	0.53 ± 0.27	-0.54 ± 0.32
<i>S. apiospermum</i>	4/≥32	0.97 ± 0.35	-0.57 ± 0.52	2/≥32	0.64 ± 0.35	-1.17 ± 0.61	0.25/16	0.27 ± 0.025	-1.21 ± 0.80
<i>S. prolificans</i>	1/4	0.65 ± 0.32	-0.77 ± 0.22	≥32/≥32	NC	NC	16/≥32	NC	NC

^a MICs and MFCs were determined in triplicate with standardized methods and are reported as median values (16). Pharmacodynamics data were generated from 24-h XTT colorimetric assays performed in triplicate. The EC₅₀ was calculated by fitting inhibition data to a four-parameter logistic model (Hill equation). All experiments were performed in triplicate. CI, confidence interval.

^b NC, not calculable due to minimal antifungal activity.

presented in Fig. 2. Amphotericin B exhibited potent antifungal activity (EC₅₀ range, 0.10 to 0.12 μg/ml) in a steep concentration inhibitory effect curve (Hill slope range, -3.25 to -4.27) against *A. fumigatus* isolates, including the itraconazole-resistant isolate *A. fumigatus* 583 (Fig. 2). Amphotericin B was somewhat less active against *A. terreus* 0925 (EC₅₀ 0.53 μg/ml) and *A. terreus* 0932 (EC₅₀ 0.36 μg/ml), although substantial reductions in hyphal viability were observed as drug concentrations approached 1 μg/ml. Similarly, amphotericin B inhibited the growth *F. solani* 002 at low concentrations (EC₅₀ 0.27 μg/ml), but showed minimal effects on the viability of *F. solani* 001 (Fig. 2), while moderate inhibitory activity against *F. oxysporum* and *Scedosporium* species with relatively higher EC₅₀s (0.41 to 0.97 μg/ml) and much more shallow concentration-effect curves (Hill slopes -0.57 to -0.88) was observed (Fig. 2).

Itraconazole was consistently less active against *Aspergillus*, *Fusarium*, and *Scedosporium* spp. than amphotericin B or voriconazole. Although effective against *A. fumigatus* 293 (EC₅₀ 0.30 μg/ml, Hill slope -2.40) and *A. terreus* isolates, itraconazole activity was attenuated for *Aspergillus fumigatus* isolates with MICs of ≥0.5 μg/ml (EC₅₀ range, 0.85 to 12.3, and Hill slope -0.56). Itraconazole demonstrated minimal activity against *Fusarium* species and *Scedosporium prolificans*, but was moderately active against *Scedosporium apiospermum*, with an EC₅₀ of 0.64 μg/ml and Hill slope of -1.17.

Voriconazole effectively inhibited the growth of *A. fumigatus* isolates (EC₅₀ 0.10 to 0.37 μg/ml, Hill slope -1.20 to -3.07), including the itraconazole-resistant isolate *A. fumigatus* 583 (Fig. 2). Voriconazole also inhibited the growth of *A. terreus* 0925 and 0932 at relatively low concentrations (EC₅₀ 0.68 and 0.10 μg/ml, respectively), although a more shallow concentra-

FIG. 1. Regression plots of the relationship of inoculum versus formazan production for test isolates at 24 h. The slopes of the regression lines were not significantly different by analysis of variance.

FIG. 2. Sigmoidal concentration-inhibitory effect curves following exposure to amphotericin B (AMB), itraconazole (ITR), and voriconazole (VOR). Symbols represent the mean plus standard deviation of experiments performed in triplicate for each drug. Lines were generated by fitting data to a four-parameter logistic model (Hill equation).

tion-response curves was observed. Voriconazole was active against *Fusarium* species (EC_{50} 0.53 to 3.3 $\mu\text{g/ml}$, Hill slope -0.54 to -0.87) with the exception of the multidrug-resistant isolate *Fusarium solani* 001 (Fig. 2). Of the three agents tested, voriconazole was the most potent against *S. apiospermum* (EC_{50} 0.27 $\mu\text{g/ml}$, Hill slope -1.2). Similar to itraconazole, minimal activity was noted for voriconazole against *S. prolificans*.

Fluorescent morbidity staining. Control cells (non-drug exposed) demonstrated minimal diffuse or no fluorescence following DiBAC staining (Fig. 3). Hyphal damage following exposure to itraconazole (2 $\mu\text{g/ml}$) was clearly evident for isolates *A. fumigatus* 293, *A. terreus* 0925 and 0932, and *S. apiospermum* but less so in other isolates. For voriconazole, pronounced hyphal damage was seen for all *Aspergillus* and *Fusarium* isolates with the exception of the multidrug-resistant isolate *F. solani* 001. At a concen-

tration of 2 $\mu\text{g/ml}$, voriconazole clearly induced hyphal damage against *S. apiospermum* but caused minimal injury to *S. prolificans*. For amphotericin B, hyphal damage was observed in all *Aspergillus* species, including *A. terreus*. Although some evidence of hyphal damage could be appreciated in *Fusarium* species, fluorescence was not as pronounced as seen with *Aspergillus* species. Consistent with the MIC/MFC and XTT viability data, considerable hyphal damage was evident with *S. prolificans* but not *S. apiospermum* following exposure of hyphae to 2 μg of amphotericin B per ml (Fig. 3).

DISCUSSION

Although host immunosuppression and early laboratory diagnosis are widely recognized as the key factors in the outcome

FIG. 3. Detection of hyphal damage by fluorescent microscopy with the cellular morbidity dye DiBAC following exposure to antifungals. Hyphae of each species were prepared after 18 h in growth medium, washed, and resuspended in culture medium containing 2 µg of the following antifungals per ml: itraconazole (ITR), voriconazole, (VOR), and amphotericin B (AMB). After 6 h of incubation, cells were washed and stained with the fluorescent morbidity dye DiBAC. Hyphae were then examined by bright-field (light boxes) and epifluorescence (dark boxes) microscopy at 400× with Nomarski optics and a fluorescein isothiocyanate (FITC) filter. Fluorescence in the dark boxes is indicative of early hyphal damage by the antifungal agent.

of opportunistic mold infections, antifungal therapy remains the most amendable day-to-day variable for improving survival in patients with invasive mycoses. As such, empirical strategies designed to hedge pharmacokinetic and pharmacodynamic uncertainties in patients (e.g., dosage escalation and combination

therapy) have quickly become a standard approach for an increasingly ill and immunocompromised patient population. Unfortunately, few preclinical studies and even fewer organized clinical trials have attempted to address issues concerning antifungal pharmacodynamics or resistance breakpoints for

invasive molds (1, 19). This is not surprising given the multitude of technical issues that complicate the in vitro measurement of antifungal activity against filamentous fungi as well as the numerous factors that confound interpretation of drug activity in patients. Nevertheless, several methods have been developed in the last 3 years that offer workable approaches for studying antifungal concentration-effect relationships in filamentous fungi.

Colorimetric assays that rely on reduction of tetrazolium salts (XTT and MTT) to colored formazan products by viable cells have long been used in the study of mammalian cell cytotoxicity (20). Meletiadiis and colleagues recently standardized an assay method that can effectively quantify mold hyphal viability formed by inocula as low as 10^2 up to concentrations of 10^5 conidia/ml following 24 h of incubation (13). With this method, the authors were able to clarify diffuse visual MIC endpoints from microdilution broth susceptibility testing and decrease the variability of antifungal concentration-effect curves for amphotericin B and itraconazole against *Aspergillus* species (13, 14). Similarly, we found that with their XTT assay, formazan production by *Aspergillus*, *Fusarium*, and *Scedosporium* spp. correlated linearly with fungal biomass after 24 h of incubation, and we were able to correlate species-specific differences in antifungal pharmacodynamics for amphotericin B, itraconazole, and voriconazole.

Several different patterns were evident from our pharmacodynamic analysis. First, itraconazole was consistently less active than either amphotericin B or voriconazole against the test isolates. The major shift towards higher EC_{50} s and shallower Hill slopes appears to occur when the MIC for the isolates approached or exceeded 1 μ g/ml. As a result, itraconazole was not active alone against *Fusarium* (MIC, 8 to 32 μ g/ml) or *S. prolificans*. Although itraconazole was active against *S. apiospermum*, the higher EC_{50} and shallower dose-response curve suggested a need for either higher itraconazole dosages or use in combination with a second agent to improve antifungal activity. Indeed, high-dose therapy and combination therapy have been recommended in the treatment of *Scedosporium* (*Pseudallescheria*) infections based on anecdotal reports of improved outcome with this approach (21).

Second, amphotericin B appeared by pharmacodynamic analysis to be remarkably active against *Aspergillus*, *Fusarium*, and *Scedosporium* species. The most notable exception was *F. solani* 001, which appeared to be cross-resistant to all of the antifungals tested. This potent activity is somewhat at odds with the sometimes high microbiological failure rates reported for amphotericin B in the treatment of these infections (21). The unusual biopharmaceutical properties and pharmacokinetics of this drug, however, may partially explain this discrepancy. Amphotericin B exhibits nonlinear, concentration-dependent binding to proteins in serum and tissue that increases with increasing drug concentrations (3). This peculiar pattern of protein binding is probably governed by the poor solubility of the amphotericin B molecule at a neutral pH (i.e., its amphoteric properties). With ultrafiltration and equilibrium dialysis, Bekersky et al. estimated that the maximum free-drug solubility of unbound amphotericin B in human plasma was 0.744 μ g/ml (3). If this represents the maximal microbiologically active fraction of amphotericin B that can be achieved in human serum or tissue, then the activity of amphotericin B for

the even more susceptible *A. terreus*, *Fusarium*, and *Scedosporium* spp. would appear to be marginal at best (see Table 1 and Fig. 2) (22).

Third, pharmacodynamic analysis of voriconazole revealed activity similar to that of amphotericin B for *A. fumigatus* (including itraconazole-resistant strains) and *A. terreus*. Voriconazole was active against most *Fusarium* isolates, but the EC_{50} s were 5- to 10-fold higher and the inhibition slopes were much shallower than seen with *Aspergillus* species, suggesting a possible need for combination therapy or higher voriconazole doses in refractory infections. Unlike amphotericin B, voriconazole is only moderately protein bound ($\approx 50\%$) and does not exhibit unlimited concentration-dependent protein binding (8). Therefore, the EC_{50} s reported for *Aspergillus*, *Fusarium*, and *S. apiospermum* are well within the range of expected free plasma voriconazole levels of 2 to 5 μ g/ml (8).

One major limitation of the XTT assay is that it primarily measures the inhibitory effects of antifungals. Similar to our findings, Meletiadiis et al. reported that EC_{50} concentrations measured by the XTT assay are, on average, two- to fourfold lower than the MICs determined by the microdilution method (13). In some but not all cases, the EC_{90} may also correlate with MFC determinations made by colony enumeration techniques (6). As such, the XTT assay appears to correlate better with MIC results but may not identify fungicidal activity.

Quantitative descriptions of fungistatic and fungicidal activity are somewhat dubious for filamentous multicellular molds, which often display both viable and dead cellular compartments on the same hyphae (5, 9). Therefore, we qualitatively assessed the degree of hyphal damage in *Aspergillus*, *Fusarium*, and *Scedosporium* cells following antifungal exposure with the fluorescent morbidity dye DiBAC (2). When fungal cells collapse with mortal injury or cell death, DiBAC is able to bind to phospholipids inside of the fungal cells and exhibit intense fluorescence under UV stimulation. Overall, DiBAC staining correlated well with the in vitro susceptibility testing results and our pharmacodynamics analyses, which found voriconazole and amphotericin B to be the more effective agents. At a fixed concentration of 2 μ g/ml, hyphae exposed to amphotericin B or voriconazole demonstrated roughly the same degree of hyphal damage, although voriconazole appeared to be more active against *Fusarium* species and *S. apiospermum* (Fig. 3). As expected from the pharmacodynamic studies, pronounced hyphal damage following exposure to 2 μ g of itraconazole per ml was seen only in *A. fumigatus* isolates with itraconazole MICs of ≤ 1 μ g/ml and *S. prolificans*.

In conclusion, we characterized species-specific differences in antifungal pharmacodynamics in vitro for amphotericin B, itraconazole, and voriconazole against *Aspergillus*, *Fusarium*, and *Scedosporium* species. Amphotericin B and voriconazole exhibited similar pharmacodynamic characteristics and were consistently more active than itraconazole against the test isolates. The XTT reduction method, when coupled with some indicator of fungicidal activity, can provide insight into the pharmacodynamics of antifungal agents for invasive molds and focus preclinical investigations for dosage escalation and combination antifungal therapy. Studies are under way to further explore the in vitro-in vivo correlation of these assays.

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