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Organism-Dependent Fungicidal Activities of Azoles

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We investigated the antifungal activities of itraconazole and voriconazole on *Aspergillus* species by time kill studies, and the results were compared with those obtained for *Candida* species. Exposure of *Aspergillus fumigatus* conidia to varying concentrations (1.25 to 10 μg/ml) of itraconazole and voriconazole resulted in cellular death; the cytocidal effect was time and concentration dependent. In contrast, no killing of *Candida albicans* occurred in the presence of itraconazole and voriconazole at concentrations as high as 10 μg/ml, although candidal growth was inhibited compared to the drug-free control. Amphotericin B (1.25 to 10 μg/ml), on the other hand, killed both *A. fumigatus* and *C. albicans*. Similar results were obtained for non-*A. fumigatus* aspergilli and non-*C. albicans Candida* species. These observations indicate that both itraconazole and voriconazole are cytocidal agents for *Aspergillus* species but not for *Candida* species, suggesting that azoles possess organism-dependent fungicidal activities.

Amphotericin B and certain members of the azole family of antifungals are the most commonly used antibiotics for the treatment of systemic fungal infections (3–6, 14). Amphotericin B acts on the cytoplasmic membrane, in particular the ergosterol component (1, 13), leading to irreversible damage of the membrane and consequent leakage of essential nutrients. The lack of a permeability barrier to essential nutrients and ions is believed to be responsible for the fungicidal activity of amphotericin B (2, 11, 12). In contrast, the azoles are known to be fungistatic agents. These heterocyclic compounds inhibit the synthesis of sterol in fungi by inhibiting cytochrome P-450-dependent 14α-lanosterol demethylase (P-450_{14DM}), which specifically removes the methyl group on C-14 of lanosterol (5, 16–18). This demethylation is an essential intermediate step in the synthesis of ergosterol, the major sterol found in fungi. The action of azoles on P-450_{14DM} of yeasts appears to be reversible; once the drug is removed, the organism recovers rapidly and functions normally. As a result of the static effect of azoles on yeasts, it is difficult to obtain clearly defined endpoints during susceptibility studies, and the so-called "trailing phenomenon" is very common (9, 15). On the other hand, the trailing phenomenon is uncommon during susceptibility tests with fungicidal agents such as amphotericin B and nystatin. During our recent studies of the susceptibility of *Aspergillus* species to voriconazole, we observed clearly defined endpoints, and prolonged incubation of the MIC tubes rarely resulted in higher MICs (8). Moreover, the minimum fungicidal concentrations were only two- to fourfold higher than the MICs. These findings suggested that voriconazole acts possibly as a fungicidal agent for *Aspergillus fumigatus* and prompted us to examine the activity of voriconazole against pathogenic yeasts and *Aspergillus* species by kill curve experiments and to compare the results with those obtained for itraconazole and amphotericin B.

Clinical isolates of *A. fumigatus* and non-*A. fumigatus* species were obtained from the Microbiology Laboratory, Detroit Medical Center, Wayne State University. Working cultures were maintained on peptone-yeast extract-glucose (PYG) (peptone, 1 g; yeast extract, 1 g; glucose, 3 g [per liter of distilled water]) agar at room temperature. The primary cultures obtained from the Detroit Medical Center were subcultured on PYG agar to assure the purity of the cultures. Long-term storage of the cultures was done as conidial suspensions in 25% glycerol at −70°C.

Working cultures of *Candida* species used in this study were grown for 48 h at 30°C on Sabouraud dextrose agar from stock cultures stored at −70°C in litmus milk (Becton Dickinson Microbiology Systems, Cockeysville, Md.). Single colonies from the 2-day-old cultures were used as the source of the inoculum for all subsequent experiments.

Conidial suspensions of various *Aspergillus* species were prepared, and the MICs of various antifungals were determined as described previously (7, 10). Briefly, fresh conidia were resuspended in PYG medium at a density of 2×10^4 conidia/ml. Twice the required concentrations of the drugs were prepared in PYG medium (0.5 ml) by serial dilution in sterile 6-ml polystyrene tubes (Falcon 2054) and inoculated with an equal volume (0.5 ml) of the conidial suspension. The tubes were incubated at 35°C for 48 h and scored for visible growth after gentle vortexing of the tubes or scraping of the walls of the tubes followed by vortexing. The MIC was defined as the lowest concentration of drug at which no visible growth occurred. The MIC determination for each isolate was repeated at least once, and the data were within ±1 dilution.

The MICs of amphotericin B, itraconazole, and voriconazole for various *Candida* species used in this study were determined by the broth microdilution method, as recommended by the National Committee for Clinical Laboratory Standards (9). The MIC was defined as the lowest concentration of drug that inhibited growth by 80% compared to the drug-free control after 48 h of incubation at 35°C.

***Aspergillus* spp.** Five milliliters of conidial suspension prepared in PYG broth (10^6 conidia/ml) was incubated at 35°C in the presence of various concentrations of amphotericin B, itraconazole, and voriconazole (0 to 10 μg/ml). At various time intervals, 0.1-ml aliquots of the conidial suspension were removed and diluted appropriately to obtain 10- to 10^4-fold dilutions, and 0.1-ml aliquots were spread in duplicate on PYG agar plates. The plates were incubated at 35°C for 48 h, and the

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TABLE 1. Susceptibilities of microorganisms tested[a]

Microorganism	Source[b]	MIC (μg/ml)[c]		
		AMB	ITZ	VCZ
A. fumigatus W73355	DMC	0.5	0.5	0.5
A. fumigatus F55064	DMC	0.5	0.25	1
A. fumigatus H52950	DMC	1	0.25	0.5
A. fumigatus T52654	DMC	0.5	0.25	0.5
A. fumigatus Z88896	DMC	1	0.5	1
Aspergillus niger S11338	DMC	4	4	1
A. niger F51729	DMC	1	4	1
A. niger I71775	DMC	1	4	1
A. niger W7884	DMC	2	0.25	0.25
A. niger T57275	DMC	2	0.5	2
Aspergillus flavus I65850	DMC	0.5	0.25	1
A. flavus I65680	DMC	8	0.25	0.5
A. flavus W69597	DMC	4	0.25	0.5
A. flavus S47511	DMC	4	0.5	0.25
A. flavus W72335	DMC	4	0.25	0.25
Aspergillus sp. M65388	DMC	0.5	0.5	0.5
Aspergillus sp. I35077	DMC	1	0.5	0.25
Aspergillus sp. R26451	DMC	2	4	0.5
C. albicans 90028	ATCC	0.02	0.031	0.015
Candida guilliermondii 9390	ATCC	0.25	0.5	0.25
Candida lusitaniae 40438	DMC	0.5	0.25	0.5
Candida parapsilosis CM205.95	DMC	0.5	0.125	0.125
Candida kefyr LK061.90	DMC	0.5	0.125	0.125
Candida stellatoidea GW575.90	DMC	0.125	0.125	0.125
Candida tropicalis 44508	ATCC	0.5	0.25	0.25
Candida glabrata 33554	ATCC	0.5	0.5	0.5

[a] Results shown are from a typical experiment. Each value represents the mean of two independent determinations. MIC determinations were repeated at least once, and the results were within ±1 dilution.
[b] DMC, Detroit Medical Center; ATCC, American Type Culture Collection.
[c] AMB, amphotericin B; ITZ, itraconazole; VCZ, voriconazole.

numbers of CFU per milliliter of conidial suspension were determined.

***Candida* spp.** Fresh 24-h-old cultures of *Candida* species prepared in PYG broth were diluted approximately 1,000-fold to obtain a cell density of 10^6 CFU per ml. One-milliliter aliquots of the diluted cultures were incubated with 0 to 10 μg of the antifungals per ml at 35°C. At various time intervals, aliquots (0.05 ml) of the drug-treated cell suspensions were removed and serially diluted (10- to 10^6-fold), and 0.1-ml amounts were spread on Sabouraud dextrose agar plates in duplicate. The plates were incubated at 35°C for 24 h, and the numbers of CFU/ml of culture were determined. A similar treatment without the drug was used as a growth control where applicable.

Itraconazole (R 51,211, batch no. STAN-9304-005-1), voriconazole, and amphotericin B (batch no. 20-914-29670) were obtained from Janssen Pharmaceutica, Beerse, Belgium; Pfizer Pharmaceuticals, New York, N.Y.; and Squibb Institute for Medical Research, Princeton, N.J.; respectively. All antifungals were dissolved in dimethyl sulfoxide at concentrations of 1 mg/ml and stored as 0.25-ml aliquots at −20°C. The frozen stocks were thawed at room temperature and gently vortexed several times to ensure that any remaining crystals were completely dissolved before use. Drug concentrations ranging from 0 to 16 μg/ml were used for MIC determinations. Comparable concentrations of dimethyl sulfoxide were tested to examine its effect on the growth of *A. fumigatus*. No detectable inhibition of growth occurred at the concentrations used.

The MICs of amphotericin B, itraconazole, and voriconazole obtained for various *Aspergillus* and *Candida* species are shown in Table 1. As shown, all of the isolates used in this study were susceptible to low concentrations of amphotericin B (MIC range, 0.02 to 4 μg/ml), itraconazole (MIC range, 0.031 to 4 μg/ml), and voriconazole (MIC range, 0.015 to 2 μg/ml).

The effects of amphotericin B, itraconazole, and voriconazole over a 24-h period on the ability of *A. fumigatus* W73355 conidia to produce colonies are shown in Fig. 1. The concentrations (1.25 to 10 μg/ml) of the drugs used for the kill curve studies were 2.5- to 20-fold higher than the MICs of the drugs for efficient killing. All three compounds at the concentrations used reduced the number of CFU with time in a dose-dependent manner, compared to the initial inoculum. For example, approximately 99% of the conidia were killed by amphotericin B at 5 μg/ml (Fig. 1A) within 24 h. Under the same conditions, itraconazole at 5 μg/ml (Fig. 1B) provided 85% killing. Approximately 95% killing was obtained with voriconazole at 5 μg/ml (Fig. 1C), suggesting that it has slightly better fungicidal activity than itraconazole but is not as efficient as amphotericin B.

Figure 2 shows the effects of exposure of *Candida albicans* to amphotericin B, itraconazole, and voriconazole over a 24-h period. In contrast to the fungicidal activities of the three drugs

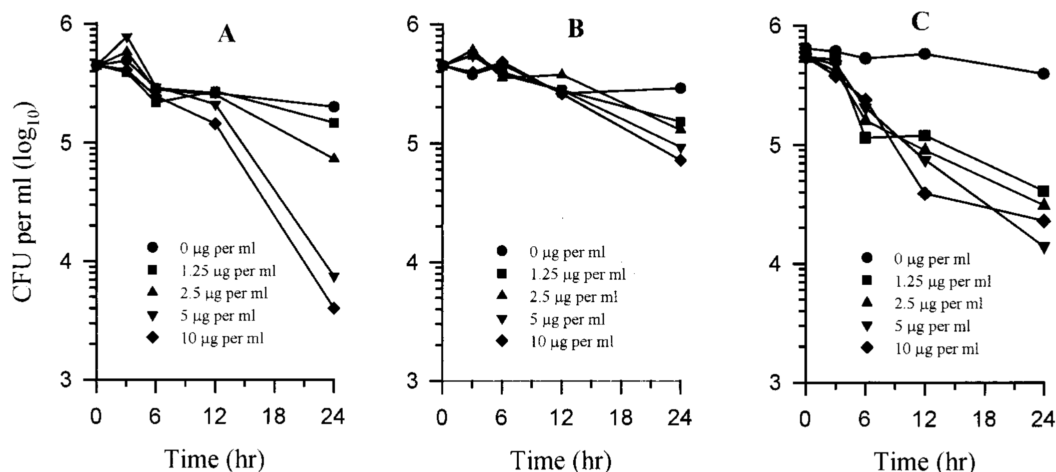

FIG. 1. Comparison of the fungicidal activities of amphotericin B (A), itraconazole (B), and voriconazole (C) against *A. fumigatus* W73355. Each point represents the mean of two independent determinations. Experiments were repeated three times with similar results; the data shown here are from a typical experiment.

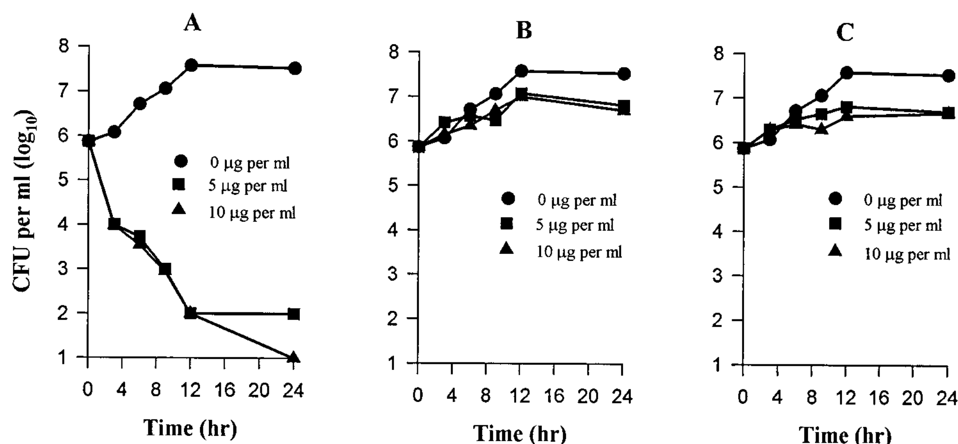

FIG. 2. Comparison of the fungicidal or fungistatic activities of amphotericin B (A), itraconazole (B), and voriconazole (C) against *C. albicans* 90028. Each point represents the mean of two independent determinations. Experiments were repeated twice with similar results; the data shown here are from a typical experiment.

against *Aspergillus*, only amphotericin B showed a reduction in CFU per milliliter with time against *Candida* species. Amphotericin B at 5 and 10 µg/ml provided ≥99.99 and 100% killing, respectively (Fig. 2A) within 24 h, whereas no killing was obtained at either 5 or 10 µg of itraconazole (Fig. 2B) or voriconazole (Fig. 2C) per ml. With both azoles, the growth of the organism after 24 h was inhibited approximately 80 to 85% compared to the drug-free control. However, there was an increase in CFU per milliliter compared to the initial inoculum.

In addition to *A. fumigatus* and *C. albicans*, we investigated the fungicidal activities of amphotericin B, itraconazole, and voriconazole on other clinically important species of *Aspergillus* and *Candida*. As shown in Table 2, non-*A. fumigatus* aspergilli examined showed ≥87% reduction in CFU per milliliter within 24 h of drug exposure. Hence, itraconazole and voriconazole were fungicidal against *A. fumigatus* and other *Aspergillus* species tested. All seven non-*C. albicans Candida* species examined were killed (≥95%) within 24 h by amphotericin B, whereas no killing was obtained with voriconazole and itraconazole. This finding demonstrated that the azoles tested have only fungistatic activity against *C. albicans* and the other yeasts examined.

Our present observations show that itraconazole and voriconazole, like amphotericin B (albeit to a lesser degree), have fungicidal activity against *A. fumigatus* and the other *Aspergillus* species examined. Shorter incubation times of conidia in the presence of higher concentrations of drugs were not very effective. This finding is not surprising considering that any killing of cells via inhibition of the sterol synthetic pathway would take longer than production of a leaky cytoplasmic membrane by direct action on ergosterol (e.g., amphotericin B). Furthermore, our studies support previous findings that the azoles have fungistatic activity against *Candida* species. Varying concentrations (including levels achievable in humans) of itraconazole and voriconazole showed time-dependent cytocidal activities against *Aspergillus* species but only cytostatic effects against *Candida* species. The exact reason(s) for such differential activities of azoles against fungi is not clear. It is possible that the sterol synthetic pathway in *Aspergillus* is essential and that inhibition of ergosterol synthesis may lead to cell death in *Aspergillus* but not in *Candida*. As fungal infections due to filamentous fungi are seen increasingly in patients with immunocompromised states, the cytocidal activities of azoles against *Aspergillus* species may be of clinical significance

TABLE 2. Fungicidal or fungistatic activities of amphotericin B, itraconazole, and voriconazole against *Aspergillus* and *Candida* species[a]

Microorganism	10^5 CFU/ml at T_0	10^5 CFU/ml at T_{24} (% change)[b]		
		AMB	ITZ	VCZ
A. fumigatus (n = 5)	8.52 ± 2.67	0.071 ± 0.091 (−99.2)	0.195 ± 0.297 (−97.7)	0.096 ± 0.055 (−98.9)
A. niger (n = 5)	4.19 ± 1.85	0.074 ± 0.097 (−98.2)	0.541 ± 0.349 (−87.1)	0.224 ± 0.140 (−94.7)
A. flavus (n = 5)	9.06 ± 4.78	0.173 ± 0.288 (−98.1)	0.317 ± 0.223 (−96.5)	0.376 ± 0.243 (−95.9)
Aspergillus sp. (n = 3)	5.57 ± 5.08	0.081 ± 0.123 (−98.5)	0.207 ± 0.284 (−96.3)	0.052 ± 0.023 (−99.1)
C. albicans	7.35 ± 0.68	0.00005 (−100)	65.3 ± 5.2 (+788.4)	50 ± 7.2 (+580.2)
C. guilliermondii	12.3 ± 4.2	0.00005 (−100)	16.3 ± 7.7 (+32.5)	19.2 ± 15.6 (+56.1)
C. lusitaniae	8.5 ± 2.12	0.028 ± 0.012 (−99.7)	20.1 ± 5.9 (+136.5)	131 ± 39 (+1,441.2)
C. parapsilosis	4.0 ± 1.4	0.129 ± 0.026 (−96.8)	12.8 ± 0.28 (+220.0)	13.2 ± 2.9 (+230)
C. kefyr	25.0 ± 2.4	0.092 ± 0.025 (−99.6)	60.5 ± 20.5 (+142)	66 ± 19 (+164)
C. stellatoidea	12.5 ± 4.2	0.00005 (−100)	84.0 ± 21.4 (+572)	108 ± 43 (+764)
C. tropicalis	11.7 ± 2.5	0.0067 (−99.9)	137 ± 65 (+1,070.9)	150 ± 37 (+1,182.1)
C. glabrata	7.0 ± 0	0.00005 (−100)	152 ± 30 (+2,071.4)	46.5 ± 23.3 (+564.3)

[a] Results shown are from a typical experiment. Each value represents the mean of two independent determinations. T_0 and T_{24} denote the times immediately prior to and 24 h after addition of the antifungal agent, respectively.

[b] Percent change is the decrease or increase from the original inoculum (T_0). AMB, amphotericin B; ITZ, itraconazole; VCZ, voriconazole. All drugs were used at 5 µg/ml.

and hold promise in the treatment of these frequently fatal infections.

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