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Voriconazole (UK-109,496) Inhibits the Growth and Alters the Morphology of Fluconazole-Susceptible and -Resistant *Candida* Species

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The effects of voriconazole on the growth, ultrastructure, and leakage of cytoplasmic materials of *Candida* species were investigated. MIC data showed that voriconazole was more active than fluconazole. Exposure of yeast to voriconazole caused growth inhibition, cell wall thinning, and cell membrane degradation. Neither cell collapse nor release of cytoplasmic materials was observed in the treated cells.

Voriconazole is a new triazole derivative that shows efficacy against a wide spectrum of fungi (4), has high oral bioavailability, is well tolerated (1), and has low systemic clearance in mouse and rat (6). All the triazole antifungal agents belong to the class of cytochrome P-450-dependent 14α-sterol demethylase inhibitors (5). The aim of the present study was to determine the effects of voriconazole on the growth and morphology of *Candida* spp.

Antifungal agents. Voriconazole and fluconazole powders were obtained from Pfizer Central Research (Sandwich, England). These agents were dissolved in distilled water to obtain stock solutions of 0.320 and 1 mg/ml, respectively. Amphotericin B (Pharma-Tech Inc., Huntington, N.Y.) was also prepared as a 3.3-mg/ml stock solution in distilled water.

Organisms and culture conditions. A fluconazole-susceptible *Candida albicans* strain, OY-2-76, and fluconazole-resistant strains, OY-12-99, JL2052, and 2038, were isolated from patients with AIDS and oropharyngeal candidiasis. *C. albicans* 572-27, *C. tropicalis* 559-6.9, *C. krusei* 630-10, and *C. glabrata* 572-26 were isolated from nonneutropenic patients with hematogenously disseminated candidiasis. *C. albicans* ATCC 36082 was purchased from the American Type Culture Collection (Rockville, Md.). *C. krusei*, *C. tropicalis*, and *C. parapsilosis* were clinical blood isolates.

Determination of the MIC_{80} and MIC_{100}. The MIC_{80}, defined as the lowest drug concentration necessary to inhibit 80% of the growth compared with that of the control, was determined by a modification of the M27-T standard method (7). The main difference was the use of serial dilution to prepare the drug solutions. Antifungal agents were serially diluted in 96-well microtiter plates (Falcon, Lincoln Park, N.J.). The final volume of the antifungal agents was 100 μl/well, and the final drug concentration ranged between 0.03 and 64 μg/ml. The MIC_{80}s were determined visually. The MIC_{100} is defined as the lowest concentration of antifungal agent at which no visible growth is observed.

Effect of voriconazole on candidal growth kinetics. *C. albicans* and *C. krusei* cells, adjusted to 10^4/ml, were used to inoculate 50 ml of RPMI 1640 medium containing different concentrations of antifungal agents (0.5×, 1.0×, and 2.0× the MIC_{80}). The cultures were incubated at 35°C with shaking. Aliquots were removed at intervals and growth was measured spectrophotometrically as previously described (2).

Effect of voriconazole on leakage of cytoplasmic materials. Leakage of intracellular constituents, estimated by the absorbance of substances at 260 nm, under the influence of voriconazole or amphotericin B was monitored by the method of Ghannoum et al. (3).

SEM. *C. albicans* cells, 10^6/ml, were grown for 24 h at 35°C in the presence or absence of 1× the MIC_{80} of either fluconazole or voriconazole. We used concentrations equivalent to the MIC_{80} instead of the MIC_{100} because the former concentration results in 80% growth inhibition, allowing the studies to be conducted on the residual cells, while the latter concentration results in no growth. Cells were fixed and processed for

TABLE 1. Susceptibility of *Candida* to voriconazole and fluconazole

Organism	Isolate	Voriconazole (μg/ml)		Fluconazole (μg/ml)	
		MIC_{80}	MIC_{100}	MIC_{80}	MIC_{100}
C. albicans (fluconazole susceptible)	OY-2-76	0.03	8	0.25	>64
C. albicans	ATCC 36082	0.03	1	0.5	>64
C. albicans (fluconazole resistant)	JL2052	0.5	2	64	>64
C. albicans	572-27	0.03	8	1	>64
C. albicans (fluconazole resistant)	OY-12-99	0.5	32	>64	>64
C. albicans (fluconazole resistant)	2038	0.5	4	64	>64
C. krusei	106	0.5	1	32	>64
C. krusei	630-10	0.125	1	16	>64
C. tropicalis	107	0.03	0.06	0.25	>64
C. tropicalis	559-6.9	0.03	0.06	0.5	>64
C. parapsilosis	108	0.03	0.125	1	4
C. glabrata	572-26	0.25	2	8	>64

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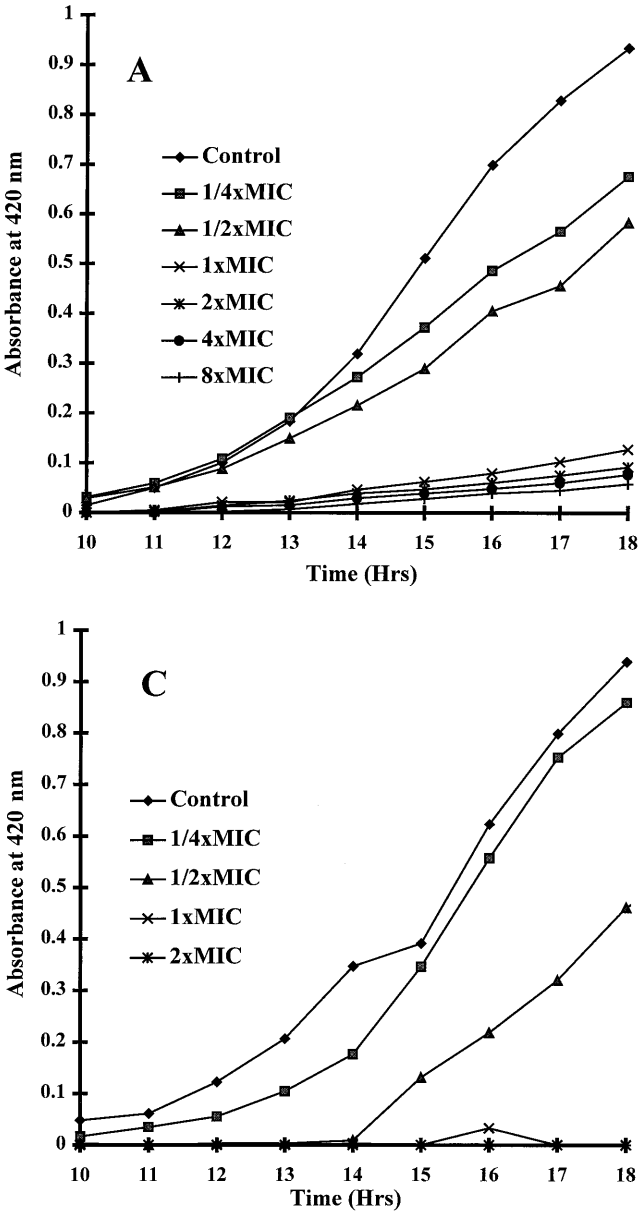

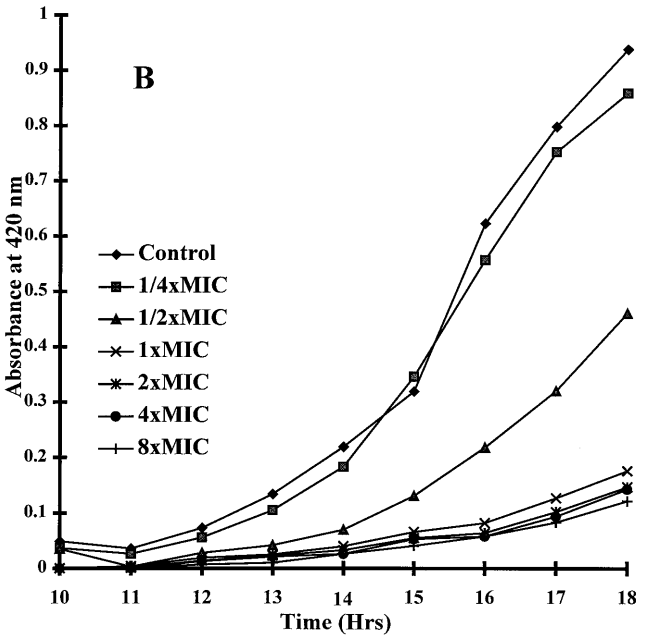

FIG. 1. Growth curves for shake cultures of *C. albicans* OY-2-76 (A), *C. albicans* OY-12-99 (B), and *C. krusei* 106 (C).

scanning electron microscopy (SEM) by the method of Ghannoum et al. (3). Samples were viewed with an S-405A (Hitachi, Tokyo, Japan) scanning electron microscope.

Transmission electron microscopy (TEM). An aliquot, 2×10^8 candidal cells, from cultures grown in the presence and absence of antifungal agents was pelleted and fixed in OsO_4 (0.1%, vol/vol). Cells were embedded in agar, cut into 1-mm^3 cubes, and suspended in 1% uranyl acetate. The cubes were embedded in Epon by graded impregnation, and ultrathin sections were cut, counterstained with lead citrate and uranyl acetate, and observed under a JEOL-100B transmission electron microscope (3).

MICs. Table 1 summarizes the MIC data for voriconazole and fluconazole for various *Candida* species. In general, the former was more active than the latter in inhibiting the growth of these species. The MIC_{80}s of voriconazole were 8- to 130-fold lower than those of fluconazole (Table 1).

Effect of voriconazole on the growth kinetics of *C. albicans* and *C. krusei*. We examined the effects of various concentrations of voriconazole on the growth rate of fluconazole-resistant *C. albicans* (OY-12-99), fluconazole-susceptible *C. albicans*, and *C. krusei*. Voriconazole showed a dose-dependent inhibitory effect on *C. albicans* as well as *C. krusei*. This was true at subinhibitory concentrations. However, since candidal growth was completely inhibited by concentrations of $\geq 1\times$ the MIC_{80} of voriconazole (Fig. 1), it is not possible to conclude that concentrations above the MIC produce greater inhibition.

Voriconazole at concentrations $\geq 1\times$ the MIC_{80} prolonged the lag phase of *C. krusei* by more than 8 h compared to the lag phase of untreated control cells (Fig. 1C), while subinhibitory concentrations of voriconazole ($0.5\times$ the MIC_{80}) prolonged the lag phase of this yeast by 4 h. Similar findings were obtained with fluconazole-susceptible and -resistant *C. albicans* (Fig. 1A and B).

Effect of voriconazole on the morphology of *C. albicans*. SEM analyses revealed that when *C. albicans* was exposed to $1\times$ the MIC_{80} of voriconazole, the morphology of the cells was altered. The yeast cells became swollen and blebs became apparent on the cell surface (data not shown). Although cells were able to bud, in some instances they were unable to divide. No cell collapse or release of cytoplasmic debris was observed following the treatment of *C. albicans* with this triazole (data not shown).

TEM. As determined by comparison to the control (Fig. 2A), treatment of *C. krusei* with voriconazole affected the outer cell envelope significantly (Fig. 2C and D). Voriconazole-treated *C. krusei* showed a pronounced separation of the cell wall and an intervening electron lucent zone between the cell wall and cytoplasm (Fig. 2C and D). Thinning of the cell wall and membrane degradation were evident (Fig. 2C). In contrast, fluconazole did not affect the morphology of this fluconazole-resistant organism, i.e., yeast cell morphology was similar to that of the untreated control (Fig. 2B).

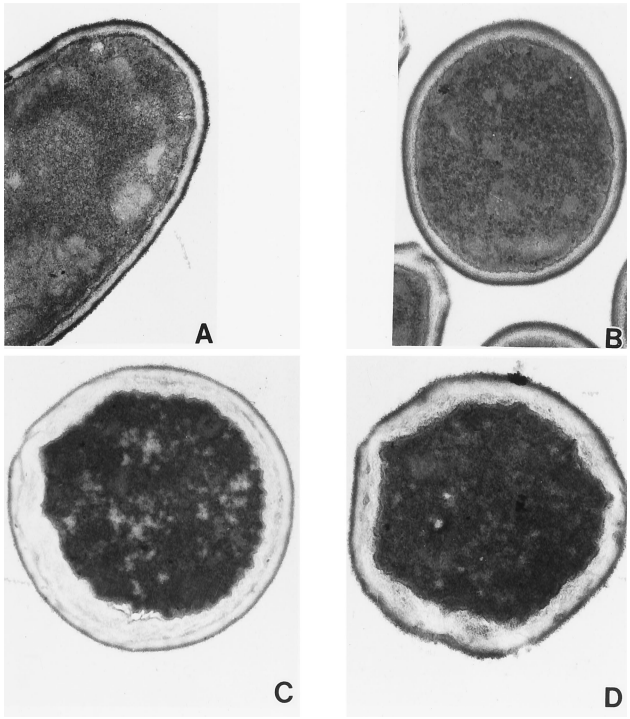

FIG. 2. Electron micrographs showing the effects of voriconazole and fluconazole on the morphology of *C. krusei*. (A) Normal untreated control; (B) fluconazole-treated organism; (C and D) voriconazole-treated yeast. Magnification, ×13,500 (all panels).

Morphological changes similar to those described for *C. krusei* were observed when fluconazole-susceptible and -resistant *C. albicans* strains were exposed to voriconazole and fluconazole. In general, the former antifungal agent was more effective than the latter in altering yeast cell wall and membrane structures (data not shown).

Effect of voriconazole on leakage of cytoplasmic materials. Irrespective of the voriconazole concentration used to treat fluconazole-susceptible *C. albicans* (up to 100 μg/ml) or the length of exposure (up to 5 h), no release of cytoplasmic materials absorbing at 260 nm into the suspension medium was observed. In contrast, candidal cells treated with amphotericin B (positive control) showed substantial leakage into the medium (data not shown). Even high concentrations of voriconazole (100 μg/ml) caused no leakage of material from *Candida* cells as determined by comparison to amphotericin B-treated cells (data not shown).

The results reported herein clearly demonstrate that voriconazole exhibits potent activity against a number of candidal strains, including those organisms known to be resistant to fluconazole, such as *C. krusei*. This antifungal agent also shows a significant effect on cell growth and ultrastructure as seen in electron microscopic examinations. In all studies performed in the present investigation, voriconazole was quantitatively more active than fluconazole.

In a separate study we have shown that the antifungal activity of voriconazole is due to the inhibition of the cytochrome P-450-dependent 14α-sterol demethylase, which is a key enzyme in the biosynthesis of ergosterol (9). The growth-inhibitory activities observed in this study were likely the result of ergosterol inhibition and amassing of methylated sterol derivatives (5).

Our TEM data, demonstrating cell wall thinning and detachment with widening of the outer cell walls of *C. krusei* and *C. albicans*, are consistent with adverse effects on chitin synthase resulting from antifungal treatment (8). Our data showed that treating *C. albicans* cells with voriconazole did not result in the release of cytoplasmic materials, suggesting that voriconazole does not exert a direct membrane-damaging effect, in contrast to amphotericin B.

In conclusion, voriconazole possesses activity against a wide spectrum of both fluconazole-susceptible and -resistant *Candida* species, inhibiting the growth of these primary opportunistic pathogens and leading to deleterious morphological modifications. This new triazole is more effective than fluconazole in its antifungal activity and promises to be a highly efficacious agent in the treatment of infections due to *Candida*.

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