Relationship between MIC and Minimum Sterol 14 α -Demethylation-Inhibitory Concentration as a Factor in Evaluating Activities of Azoles against Various Fungal Species

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The minimum growth-inhibitory concentrations (MICs) of azole antifungals were compared to their minimum sterol 14 α -demethylation-inhibitory concentrations (MDICs) for clinical fungal isolates. The ascomycetous *Candida* yeasts tested were clearly divided into two groups: group I, consisting of *C. albicans*, *C. tropicalis*, and *C. lusitanae*, had MICs that were much higher than the MDICs, whereas group II, comprising *C. glabrata*, *C. parapsilosis*, *C. guilliermondii*, and *C. krusei*, had MICs that were approximately equal to the MDICs. In the ascomycetous fungi *Aspergillus fumigatus* and *Sporothrix schenckii*, the MICs were indistinguishable from the MDICs. In the basidiomycetous fungi *Cryptococcus (Filobasidiella) neoformans*, *C. curvatus*, and *Trichosporon asahii*, the MICs and the MDICs were practically identical. These results support the notion that there are two distinct classes of fungi differing in their degree of tolerance to sterol 14 α -demethylation deficiency. These findings have significant implications for both fungal physiology and antifungal chemotherapy.

Azole antifungal agents are known to inhibit the activity of cytochrome P450_{14DM}, which catalyzes the 14 α -demethylation of sterols in ergosterol biosynthesis (21). As a consequence, cells treated with those drugs accumulate 14 α -methylated sterols in their membranes in place of ergosterol. A notable feature of this change in sterol composition is that it may or may not inhibit cell growth depending on the fungus involved. Thus, our previous work has demonstrated that under conditions of 14 α -demethylation inhibition, cells of *Candida albicans*, *C. tropicalis*, *C. guilliermondii*, and *C. kefyr* can proliferate in vitro, while those of *C. krusei*, *C. glabrata*, and *C. parapsilosis* cannot (18). Unfortunately, however, it has remained unclear whether this characteristic varies within each species due to the small number of strains examined.

In vivo data, however, indicate that azoles are therapeutically effective in *C. albicans* infection due to the inhibition of 14 α -demethylation (11). This could be because 14 α -demethylation-deficient cells are more vulnerable than normal cells to killing by reactive oxygen species (16) or phagocytes (3, 4, 5, 7, 20).

The most important implication of the above-mentioned findings is that the minimum sterol 14 α -demethylation-inhibitory concentration (MDIC), rather than the minimum growth-inhibitory concentration (MIC), may be of primary importance

for the clinical use of azole drugs. Theoretically at least, if the MIC of a drug is higher than its MDIC for a certain fungus, the drug may suppress infections it causes even at a serum concentration below the MIC (but above the MDIC) as long as host defenses are unimpaired. It is therefore necessary to collect more information about the relationship between MIC and MDIC. In the present study, we provide such data for a number of clinical isolates, including not only *Candida* species but also other ascomycetous and basidiomycetous fungi.

MATERIALS AND METHODS

Fungal strains. Ninety-five clinical isolates of yeast-like fungi, recovered from the bloodstream of patients with fungal infections, consisted of 26 strains of *C. albicans*, 17 strains of *C. tropicalis*, 8 strains of *C. glabrata*, 10 strains of *C. parapsilosis*, 21 strains of *C. guilliermondii*, 9 strains of *C. krusei* (of which 4 are laboratory strains), 2 strains of *C. lusitanae* (1), 2 strains of *Cryptococcus (Filobasidiella) neoformans*, 2 strains of *Cryptococcus curvatus* (8), and 2 strains of *Trichosporon asahii* (19). These isolates were identified presumptively by colony morphology on CHROMagar *Candida* (Becton Dickinson), chlamydospore formation on cornmeal agar, and sugar assimilation patterns with the API ID32C system (BioMerieux SA, Marcy-l'Etoile, France). Species identification was confirmed by direct sequencing of the D1-D2 region of 26S rRNA (6). Five strains of *Aspergillus fumigatus* isolated from animals were provided by Rui Kano of the School of Veterinary Medicine, Nippon University. *Sporothrix schenckii* IFM41598, originally isolated from a patient with sporothrichosis, was provided by Kazuko Nishimura of Chiba University.

MIC and MDIC determination. We have previously devised a simple method capable of measuring the MDIC of an azole drug for those fungi whose viability is unaffected by sterol 14 α -demethylation deficiency, e.g., *C. albicans* (17, 18). The method is based on the fact that 14 α -demethylation deficiency, whether caused by an azole or another mechanism, makes the fungal cells susceptible to growth inhibition by acetate added to the growth medium, probably due to an

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TABLE 1. MIC^Ys and MDICs of azoles for various fungi^a

Species ^b	No. of strains	MIC ^Y (μg/ml)			MDIC (μg/ml)		
		FLC	KTC	ITC	FLC	KTC	ITC
<i>Candida albicans</i> (26)	22	>200	>13	>13	0.4–0.8	<0.012	<0.012
	4	>200	>13	>13	0.8	<0.012	0.05
<i>C. tropicalis</i> (17)	10	>200	>13	>13	3.1	<0.012	0.05
	6	>200	>13	>13	0.2	<0.012	0.025
	1	>200	6.3	>13	1.6	0.1	0.1
<i>C. lusitanae</i> (2)	2	>200	13	>13	0.79	<0.012	<0.012
<i>C. glabrata</i> (8)	7	>200	>13	>13	>200	>13	>13
	1	100	>13	>13	100	>13	>13
<i>C. krusei</i> (9)	7	200	1.6–3.1	0.4–0.8	200	1.6–3.1	0.2–0.8
	2	100	1.6	0.4	100	0.8	0.2
<i>C. guilliermondii</i> (21)	9	200	13	>13	200	13	>13
	8	100	6.3–13	>13	100	13	>13
	2	50	0.2	0.4	50	0.1	0.2
	2	25	0.2	3.1	25	0.1	1.6
<i>C. parapsilosis</i> (10)	5	1.6	<0.012	<0.012	0.8	<0.012	<0.012
	3	1.6–3.1	0.025	0.025	1.6–3.1	0.012	0.025
	2	3.1	<0.012	0.012	1.6	<0.012	<0.012
<i>Cryptococcus neoformans</i> (2)	2	13	0.05–0.1	0.05	6.3–13	0.025–0.05	0.025
<i>C. curvatus</i> (2)	2	13	0.4–0.8	0.2–0.4	6.3–13	0.4–0.8	0.1–0.2
<i>Trichosporon asahii</i> (2)	2	13	1.6	0.4	13	0.8	0.2
<i>Aspergillus fumigatus</i> (5)	5	200	1.6–3.1	0.025–0.05	200	0.8	0.013

^a MIC^Y, minimum growth-inhibitory concentration measured in YEPG; MDIC, minimum sterol 14α-demethylation-inhibitory concentration in YEPG-Ac. See Materials and Methods for details. FLC, fluconazole; KTC, ketoconazole; ITC, itraconazole.

^b The number in parenthesis is the total number of each species studied.

increase in permeability of the cell membrane (17). Thus, for fungi of this class the MIC of an azole as determined in acetate-supplemented medium (MIC_{Ac}) is lower than its MIC measured in acetate-free medium and is similar to its MDIC, as estimated by sterol profiling with thin-layer chromatography; hence, the relationship was MIC > MIC_{Ac} = MDIC (18). In contrast, those fungi which are unable to tolerate 14α-demethylation deficiency, e.g., *C. krusei*, are characterized by the formula MIC = MIC_{Ac} = MDIC (18). Incidentally, acetate has no effect on sterol demethylation or growth rate at the concentration used. In practice, the determination of MIC and MDIC was carried out as follows. Twofold dilutions of the test drug were made in yeast extract-peptone-glucose (YEPG) medium consisting of 1% yeast extract, 2% polypeptone, and 2% glucose (for MIC^Y, which means MIC measured in YEPG) or YEPG supplemented with 0.24 M sodium acetate (YEPG-Ac) (for MDIC). A 0.1-ml portion of each dilution was mixed in a microplate well with 0.1 ml of a cell suspension in the same medium containing approximately 10⁵ CFU. The plates were incubated at 37°C for 2 days with shaking. The lowest drug concentration that gave a prominent decrease in turbidity was taken to represent the MIC^Y or MDIC of the drug. It should be noted that the MIC^Y obtained in this way is not equivalent to the MIC determined by the routine method described by the National Committee for Clinical Laboratory Standards (9). In the case of *S. schenckii*, the high susceptibility of this fungus to acetate made the use of the above method for MDIC determination inadequate. Hence, direct estimation of MDIC by sterol profiling with thin-layer chromatography was carried out as previously described (17).

RESULTS

Candida species. Our previous study demonstrated that the strains of *Candida* species examined were divided into two groups with respect to the relationship between MIC and MDIC (18). However, the small number of test strains made it uncertain whether this characteristic was species specific. In the present work, we studied this dichotomy with a much larger number of strains (Table 1). For strains of *C. albicans* and *C. tropicalis*, the MIC^Y (>200 μg/ml for fluconazole) was much higher than the MDIC (0.2 to 3.1 μg/ml), as previously reported. In addition, strains of *C. lusitanae* were found to belong to this group. On the other hand, the MIC^Y was essentially equal to the MDIC for strains of *C. glabrata*, *C. parapsilosis*, and *C. krusei*, in agreement with the previous

results (18). Only *C. guilliermondii* exhibited an intraspecific heterogeneity: strain IFO0454, used in the previous studies, is a member of the former group, while the 21 strains tested in this work were found to belong to the latter group. These results strongly suggest that intraspecific variation in this MIC-MDIC characteristic is rare. In other words, this characteristic can be regarded as species specific for *Candida* species.

Basidiomycetous yeast species. For all strains of the basidiomycetous yeasts *C. neoformans*, *C. curvatus*, and *Trichosporon asahii* tested, the MIC^Y was found to be comparable to the MDIC (Table 1).

Filamentous fungi. We successfully applied the YEPG-Ac method for MDIC determination to five strains of the ascomycetous mold *A. fumigatus*, and we found for all of them that the MIC^Y was comparable to the MDIC (Table 1). The ascomycetous fungus *S. schenckii* is dimorphic and grows either as filaments at 25°C or as yeasts at 37°C (10). Because the growth of this fungus turned out to be severely inhibited by acetate even in the absence of azoles, the use of YEPG-Ac medium for MDIC determination was considered inadequate. We therefore studied growth and sterol composition of cells of this fungus cultured aerobically in YEPG medium at 25°C in the presence of various concentrations of ketoconazole. The azole drug inhibited by more than 80% both cell growth and sterol 14α-demethylation at a concentration of 1.25 μg/ml and higher compared to the drug-free control cells (Fig. 1). The same results were obtained with yeast cells grown at 37°C (data not shown). Thus, the MIC^Y and MDIC of ketoconazole were shown to be similar for this organism. In addition, the MIC^Y estimated by optical density was compatible with morphological observations. While cells grew in typically filamentous form without ketoconazole, the presence of the drug at 1.25 μg/ml completely inhibited their growth.

FIG. 1. Thin-layer chromatography profiles of sterols from *S. schenckii* cells grown in the presence of various concentrations of ketoconazole. Cells were grown with shaking in YEPG (see Materials and Methods) at 25°C for 3 days. The cell yields were assessed by optical density, and the cellular lipids were extracted by the method of Bligh and Dyer (2). Numbers below and above each lane are ketoconazole concentration (in micrograms/milliliter) and relative cell yields (in percent), respectively. Thin-layer chromatography analysis was carried out as described previously (12, 14) with the solvent system of diethyl ether/acetic acid/petroleum ether (100:3:97). Identification of sterols: a, 4,4',14-methylated sterols; b, 4,14-methylated sterols; c, ergosterol; d, phospholipids; e, polar sterol (14).

DISCUSSION

The results of the present study are consistent with the notion that fungi can be divided into two types based on their tolerance to deficiency in sterol 14 α -demethylation. Most, if not all, species appear to be homogeneous with respect to this trait, and the species for which the drug MIC and MDIC are comparable to each other appear to be more common than those for which the in vitro MIC is higher than the MDIC. The mechanism underlying the difference in the degree of tolerance to 14 α -methylated sterols is unknown at present. One possibility is that different degrees of tolerance may be due to the effect of the 14 α -methyl group on the function of one or more membrane proteins that interact with sterols in membranes.

C. albicans and *C. tropicalis*, for which the MIC^Y is higher than the MDIC, are both important opportunistic pathogens, frequently causing systemic mycoses in compromised hosts. Our previous studies have shown that *C. albicans* cells exhibit increased sensitivity to various antifungal chemicals when their membranes contain 14 α -methylated sterols in place of ergosterol, probably due to enhanced membrane permeability (13, 15). This observation immediately suggests the possibility that a combination of an azole and an antifungal from another class could act synergistically on fungal cells at least of this type. It is hoped that this may open up a new avenue of antifungal chemotherapy.

The values of MDICs for *Candida* species are similar to MICs obtained by the standard NCCLS RPMI method (9, 18) and correlate with the effectiveness of antifungal agents in vivo. We are therefore currently investigating the wider application of the simple MDIC methodology to the azole susceptibility testing of filamentous fungi, such as *Aspergillus* spp.

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