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Fluconazole Tolerance in Clinical Isolates of *Cryptococcus neoformans*

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Eleven isolates of *Cryptococcus neoformans* were investigated to determine the biochemical basis of their tolerance to fluconazole. The MICs of fluconazole for three isolates with low-level resistance were 3- to 6-fold higher than those for sensitive isolates, while the MICs for four isolates with high-level resistance were 100- to 200-fold higher than those for sensitive isolates. The level of ergosterol present in the isolates varied, and those which had relatively low levels of ergosterol were resistant to amphotericin B. Changes in the affinity of the target enzyme (sterol 14α-demethylase) and decreases in the cellular content of fluconazole seemed to be responsible for the resistance in isolates with low-level and high-level resistance, respectively.

Advances in medicine that alter the immune status of patients and an increase in the number of patients with immunodeficiency induced by human immunodeficiency virus infection have been major causes for a significant increase in the incidence of fungal infections in humans over the past decade (1, 2). Opportunistic fungal pathogens such as *Candida albicans*, *Cryptococcus neoformans*, and *Aspergillus fumigatus* have accounted for most of the mycotic infections in immunocompromised individuals, and these infections often become life-threatening (3). *C. neoformans* causes meningoencephalitis in approximately 6 to 8% of AIDS patients (22).

Although amphotericin B (a polyene antifungal antibiotic) and fluconazole (a triazole antifungal agent) are currently available for the treatment of cryptococcal meningitis, the latter drug became the drug of choice because of severe side effects such as nephrotoxicity associated with the use of amphotericin B (4). Fluconazole, like other azole antifungal drugs, exerts its antifungal activity by interfering with ergosterol biosynthesis by inhibiting sterol 14α-demethylase ($P450_{14dm}$), leading to membrane disorganization, leakage of essential cytoplasmic materials, and growth arrest (for a review, see reference 9). The azole moieties of these drugs ligate to the ferric iron of the heme group of the target enzyme ($P450_{14dm}$), while the hydrophobic portions of the drugs interact with the substrate binding region of the apoprotein (18, 19).

Long-term usage of fluconazole for the treatment of fungal infections in AIDS patients leading to the emergence of azole resistance has been documented in several studies (for a review, see reference 8), and therefore, an understanding of drug resistance is valuable to designing antiresistance strategies when developing new drugs. Biochemical studies with azole-resistant mutants of several plant as well as animal fungal pathogens have demonstrated that there are at least two mechanisms of resistance: alterations in the enzymes involved in the ergosterol biosynthesis and changes in the intracellular accumulation of azoles (for a review, see reference 9). However, the biochemical basis for azole drug resistance in *C. neoformans* is relatively poorly understood.

Biochemical studies were undertaken to characterize the variation in fluconazole tolerance in 11 isolates of *C. neoformans* by studying the susceptibilities of these isolates to fluconazole, changes in sterol composition, sterol synthesis in response to fluconazole, sensitivity of $P450_{14dm}$ to fluconazole, and the intracellular concentration of the drug.

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MATERIALS AND METHODS

Strains. Strains 93-1703, 93-2638, 93-1440, 93-1416, 93-2527, 93-2579, 94-81, 94-179, and A18 were isolated from the cerebrospinal fluid of patients; strain 94-8 was isolated from the bronchial wash of a patient; and strain B4500 was a laboratory strain (12). After identification, the clinical strains were stored at San Antonio, Tex., but clinical details about the isolates were not available. Strains were maintained by culturing them regularly on YEPD agar (2% [wt/vol] Difco agar, 2% [wt/vol] glucose, 2% [wt/vol] Difco peptone, and 1% [wt/vol] Difco yeast extract) plates.

Chemicals. All chemicals were purchased from Sigma Chemical Company (Poole, United Kingdom) unless otherwise indicated. Fluconazole was obtained from Pfizer (Sandwich, United Kingdom). [2-^{14}C]mevalonate, dibenzethylenediamine salt (specific activity, 53 mCi/mmol), was purchased from Amersham, and [^{14}C]fluconazole (specific activity 22 μCi/mg) was a gift from Pfizer. Fluconazole at 1 μM corresponds to 0.31 μg of fluconazole per ml. Stock solutions of fluconazole (1 mM) and amphotericin B (10 mg/ml) were made in dimethyl sulfoxide.

MIC tests. The cells obtained from culture plates that had been incubated for 2 days at 37°C were resuspended in YEPD medium at 10^5 cells/ml, and 2 ml of the cell suspension was incubated with various concentrations of fluconazole or amphotericin B in a 60-ml sterilin container for 2 days at 37°C and with shaking at 150 rpm. Growth was assessed by measuring cell counts to determine the MICs of fluconazole and amphotericin B (7). The tests were carried out in triplicate.

Sterol isolation and analysis. Cells harvested from a 100-ml culture were resuspended in 3 ml of methanol–2 ml of 60% KOH–2 ml of 0.5% (wt/vol) pyrogallol dissolved in methanol and were saponified by heating at 90°C for 1 h. Nonsaponifiable sterols were extracted from the saponified mixture thrice by using 5 ml of hexane each time (24). The extracts were pooled, evaporated to dryness under nitrogen gas, and redissolved in 100 μl of toluene and 20 μl of bis(trimethylsilyl)trifluroacetamide. After silylation for 1 h at 60°C, sterols were analyzed by gas chromatography-mass spectrometry (VG 12-250; VG BIOTECH) by using split injection with a split ratio of 20:1. Sterols were identified by comparing relative retention times and mass spectra to the previously published data (14, 17).

Cell extract preparation. *C. neoformans* isolates were grown in 1 liter of YEPD medium in a 2-liter flask at 37°C and with shaking at 150 rpm. Cells were harvested when the cell density was 10^8 cells per ml, washed once, and resuspended in 50 ml of 100 mM potassium phosphate buffer (pH 7.5) containing 20% (wt/vol) glycerol, mixed with 20 g of glass beads (0.4 to 0.5 mm in diameter), and homogenized with a Braun disintegrator (Braun GmbH, Mesungen, Germany) operating at 4,000 rpm by four 30-s bursts with liquid carbon dioxide cooling. The homogenate was centrifuged at 1,500 × *g* to obtain cell extracts (21). The protein content in the cell extracts was estimated by using the bicinchoninic acid method (Sigma).

Sterol biosynthesis in cell extracts. Fluconazole inhibition of the $P450_{14dm}$ of *C. neoformans* was studied by assessing the sterol biosynthesis in cell extracts by using [2-^{14}C]mevalonate as described previously (21).

TABLE 1. MICs of fluconazole and amphotericin B for growth, IC_{50}s of fluconazole for cell-free C-14 desmethyl sterol biosynthesis, and cellular content of [^{14}C]fluconazole in *C. neoformans* isolates[a]

Isolate[b]	Fluconazole MIC (μM)	Amphotericin B MIC (μg/ml)	IC_{50} (nM)[c]	[^{14}C]fluconazole (pmol/10^9 cells)[d]
A18^s	1.0	1.0	10.0 ± 0.9	8.02 ± 0.21
B4500^s	1.0	1.0	9.2 ± 0.6	10.13 ± 0.70
94-81^{lr}	6.4	1.0	33.3 ± 1.7	11.16 ± 0.21
93-2527^{lr}	6.4	1.0	33.0 ± 2.6	14.59 ± 0.16
93-2579^{lr}	3.2	0.8	22.0 ± 0.9	10.31 ± 0.60
94-8^s	1.3	10.0	13.0 ± 1.4	17.16 ± 0.35
93-2638^s	1.6	10.0	15.0 ± 0.7	15.88 ± 0.67
93-1416^{hr}	128.0	10.0	9.0 ± 0.8	0.82 ± 0.09
94-179^{hr}	256.0	5.0	11.5 ± 1.6	0.80 ± 0.12
93-1703^{hr}	210.0	1.0	15.0 ± 1.1	0.46 ± 0.07
93-1440^{hr}	128.0	1.0	8.0 ± 1.0	0.68 ± 0.10

[a] The results are the means ± standard deviations of three experiments.
[b] s, sensitive to fluconazole; lr, low-level resistance to fluconazole; hr, high-level resistance to fluconazole.
[c] Cell-free C-14 desmethyl sterol biosynthesis.
[d] Cellular content of [^{14}C]fluconazole.

Measurement of fluconazole uptake. Cellular levels of [^{14}C]fluconazole in the sensitive and tolerant isolates were measured by using a filter-based assay described previously (21). The cells obtained from the culture plates incubated at 37°C for 3 days were washed once, resuspended in 100 mM phosphate buffer (pH 7.0) at a cell density of 10^9 cells per ml, and incubated with 10 μM [^{14}C]fluconazole at 37°C and 150 rpm. The content of fluconazole in the cell pellets reached a maximum by 1 h, and therefore, the samples were harvested after 2 h of exposure to the drug. The cells were washed three times with 10 ml of 100 μM unlabelled fluconazole at room temperature after filtering them onto Whatman GFC filters. The filters were dried and transferred to a scintillation vial containing 10 ml of scintillation fluid, and the radioactivity was measured with a Philips 4700 scintillation counter. Control experiments with autoclaved cells were done in order to establish the amount of drug that bound to the cells. In all the isolates the amount of drug that bound to the cells did not exceed 10% of the incorporated activity.

RESULTS AND DISCUSSION

The MICs of fluconazole and amphotericin B for *C. neoformans* isolates were determined in this study (Table 1). Three isolates (94-81, 93-2527, and 93-2579; isolates with low-level resistance) exhibited 3- to 6-fold more tolerance to fluconazole than the sensitive isolates, and four isolates (isolates 93-1416, 94-179, 93-1703, and 93-1440; isolates with high-level resistance) exhibited 100- to 200-fold more tolerance to fluconazole than the sensitive isolates. Two of the high-level resistant isolates showed cross-resistance (5- to 10-fold) to amphotericin B. In addition, two isolates (isolates 94-8 and 93-2638) which were sensitive to fluconazole also exhibited a 10-fold increased resistance to amphotericin B.

The sterol analysis of the isolates in the presence or in the absence of fluconazole was carried out to determine the cause for the drug tolerance. When grown in the absence of fluconazole, a considerable variation in the sterol composition of the *C. neoformans* strains was observed. Two sensitive isolates (isolates A18 and B4500), three low-level-resistant isolates (isolates 94-81, 93-2527, and 93-2579), and two high-level-resistant isolates (isolates 93-1703 and 93-1440) were found to contain ergosterol as their predominant sterol (Table 2). In contrast, the ergosterol levels in the isolates which were resistant to amphotericin B were comparatively low. This result was expected because amphotericin B acts by interacting with the ergosterol (16). However, we showed in recent studies the existence of amphotericin B resistance in some strains of *C. albicans* and *C. neoformans* containing normal levels of ergosterol, and we do not yet know the actual cause for the resistance in these isolates (7, 21). In isolates 94-8 and 93-2638, a reduction in the ergosterol level correlated with an increase in ergosta-8-eno. Its accumulation is the indication for a defect in sterol $\Delta^{8\rightarrow7}$-isomerase, one of the enzymes involved in ergosterol biosynthesis. The decrease in ergosterol levels in isolates 93-1416 and 94-179 was associated with the accumulation of ergosta-7,22-dienol. Accumulation of this sterol is characteristic of a lesion in the sterol $\Delta^{5,6}$-desaturase, which is involved in ergosterol biosynthesis (10).

Exposure to the MIC of fluconazole produced a decrease in the ergosterol level to below 20% in all the isolates (Table 3). All the treated isolates accumulated obtusifolione and eburicol at very high levels. The presence of high levels of the latter sterol and the former sterol are indicative of the inhibition of $P450_{14dm}$ and 3-ketosteroid reductase (which is involved in ergosterol biosynthesis), respectively. This result suggests the inhibition of 3-ketosteroid reductase along with $P450_{14dm}$ by azoles directly or by retention of the C14α-methyl group in the substrate, a result in agreement with previous observations (7, 20). In yeast and *Candida* spp., accumulated lanosterol and eburicol (the substrate for $P450_{14dm}$) in the presence of azoles undergoes subsequent steps in ergosterol biosynthesis and finally is converted into 14α-methyl 3,6-diol by sterol $\Delta^{5,6}$-desaturase. In these organisms azole resistance results from the defect in sterol $\Delta^{5,6}$-desaturase because of the accumulation of 14α-methyl fecosterol, which is capable of supporting growth, instead of 14α-methyl 3,6-diol in the presence of azoles drugs (10,23). However, from recent studies on the mode of action of azoles on *C. neoformans* we predicted that this kind of resis-

TABLE 2. Sterol compositions of 24-h-old cultures of *C. neoformans*

Sterol	% Total sterols for isolate[a]:										
	A18^s	B4500^s	94-81^{lr}	93-2527^{lr}	93-2579^{lr}	94-8^s	93-2638^s	93-1416^{hr}	94-179^{hr}	93-1703^{hr}	93-1440^{hr}
Ergosta-tetraenol	ND	2.4	1.5	0.7	0.9	3.5	2.7	ND	3.6	2.9	9.6
Ergosterol	54.5	65.4	71.1	53.3	48.0	27.1	31.3	8.6	41.1	96.5	58.2
Ergosta-7,22-dienol	3.4	4.0	ND	1.3	ND	7.4	6.9	68.0	31.2	ND	3.3
Ergosta-8-enol	ND	3.6	1.8	ND	9.8	51.8	44.2	ND	ND	ND	ND
Ergosta-7-enol	13.8	14.7	ND	2.8	ND	ND	ND	23.4	21.6	ND	6.1
Eburicol	ND	4.2	23.3	37.5	36.0	3.5	ND	ND	1.2	ND	15.7
4-Methyl fecosterol	10.1	ND	ND	1.6	1.5	1.6	1.8	ND	0.3	0.6	2.4
Ignosterol	ND	ND	0.5	ND	ND	1.5	2.1	ND	ND	ND	ND
Lanosterol	14.6	ND	ND	2.3	ND	1.2	1.5	ND	ND	ND	3.2
Episterol	ND	ND	ND	ND	3.8	1.2	ND	ND	0.4	ND	ND
Unidentified sterols	3.6	5.7	1.8	0.5	9.9	1.2	9.5	ND	0.6	ND	1.5

[a] s, sensitive to fluconazole; lr, low-level resistance to fluconazole; hr, high-level resistance to fluconazole; ND, not detected.

TABLE 3. Sterol compositions of the various isolates treated with fluconazole at the MIC

Sterol	% Total sterols for isolate[a]:										
	A18^{s}	B4500^{s}	94-81^{lr}	93-2527^{lr}	93-2579^{lr}	94-8^{s}	93-2638^{s}	93-1416^{hr}	94-179^{hr}	93-1703^{hr}	93-1440^{hr}
Ergosterol	15.0	8.3	9.9	17.3	4.4	10.5	11.0	ND	3.7	18.7	12.4
14α-Methyl fecosterol	ND	ND	ND	ND	ND	ND	ND	2.4	4.5	ND	ND
Obtusifolione	14.9	20.7	30.4	20.5	19.5	7.7	32.2	7.4	7.0	23.4	4.3
4,4-Dimethyl-ergosta-8,14,24(28)-trienol	1.0	ND	3.4	0.2	6.4	ND	3.7	ND	ND	ND	1.9
Obtusifoliol	2.1	1.7	4.0	1.7	5.6	11.4	9.0	16.4	12.2	2.7	2.4
Eburicol	67.0	69.0	52.3	60.3	64.1	70.4	42.6	70.3	72.6	47.8	78.6
Unidentified sterols	ND	0.3	ND	ND	ND	ND	1.6	3.5	ND	7.4	0.4

[a] s, sensitive to fluconazole; lr, low-level resistance to fluconazole; hr, high-level resistance to fluconazole; ND, not detected.

tance cannot occur in *C. neoformans* due to the inhibitory effects on 3-ketosteroid reduction, which precedes sterol $\Delta^{5,6}$ desaturation in the ergosterol biosynthetic pathway (7, 20). The prediction was confirmed by this study; isolates 93-1416 and 94-179 (sterol $\Delta^{5,6}$-desaturase mutants) accumulated obtusifolione instead of 14α-methyl fecosterol in the presence of fluconazole, suggesting that their resistance was not due to the defect in sterol $\Delta^{5,6}$-desaturase. In other studies, sterol $\Delta^{8\rightarrow7}$-isomerase mutants were not resistant to azole drugs because the sterol that accumulated in azole-treated cultures of these mutants is not capable of supporting growth, although these strains were resistant to amphotericin B (11). In the *C. neoformans* isolates studied here, a similar kind of phenotype was observed for the sterol $\Delta^{8\rightarrow7}$-isomerase mutants.

The inhibition of $P450_{14dm}$ by fluconazole was tested by measuring the incorporation of [2-^{14}C]mevalonate into C-14 desmethylated sterols in cell extracts. Cell extracts of all *C. neoformans* isolates were active in incorporating [2-^{14}C]mevalonate into C-14 desmethylated sterols. The half inhibitory concentrations (IC_{50}s) of fluconazole for the C-14 desmethylated sterols are presented in Table 1. This result suggests that the sensitivity of $P450_{14dm}$ of isolates with high-level resistance to fluconazole was comparable to that of the sensitive isolates. In contrast, the $P450_{14dm}$ of low-level-resistant isolates was relatively less sensitive to fluconazole, and the reduction in the sensitivity correlated with their tolerance to fluconazole in vitro. Alteration in the susceptibility of the target enzyme to fluconazole could be due to changes in either the levels of the enzyme or the affinity of the enzyme to fluconazole. Azole resistance due to the reduced susceptibility of $P450_{14dm}$ was also observed in isolates of *C. neoformans* from AIDS patients and in *Ustilago maydis* (6, 12).

The cellular content of fluconazole in the 11 isolates of *C. neoformans* was investigated by using radiolabelled fluconazole (Table 1). The cellular content of fluconazole in the sensitive and low-level-resistant isolates was approximately 8 to 17 pmol per 10^9 cells. In contrast, the intracellular content of fluconazole in high-level-resistant isolates of *C. neoformans* was only about 0.4 to 0.8 pmol per 10^9 cells. The 10- to 20-fold reduction in the intracellular accumulation of fluconazole in the high-level-resistant isolates correlated, at least partially, with their tolerance to fluconazole. The correlation between azole resistance and reduced intracellular accumulation of drug was reported in several recent studies (5, 7, 13, 15, 21). The involvement of multidrug resistance transporters in azole resistance was shown in *C. albicans* and was suggested for *Candida glabrata* (13, 15), and similar kinds of transporters may be involved in reducing the cellular content of drug in the high-level-resistant isolates of *C. neoformans*. In conclusion, reduced susceptibility of $P450_{14dm}$ was involved in low-level fluconazole resistance and the reduced cellular content of drug appeared to account for high-level fluconazole resistance in *C. neoformans* in the strains described here.

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