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Reduced Accumulation of Drug in *Candida krusei* Accounts for Itraconazole Resistance

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Due to intrinsic resistance *Candida krusei* is emerging as a systemic pathogen in AIDS patients undergoing fluconazole therapy, but acquired resistance to itraconazole has not been studied biochemically. We report here studies on the basis for azole resistance and sterol composition in *C. krusei*. An itraconazole-resistant isolate showed reduced susceptibility to azole drugs in in vitro growth inhibition studies. Accumulation of 14α-methyl-3,6-diol under azole treatment was associated with growth arrest. In vitro ergosterol biosynthesis and type II binding studies suggested no alteration in the affinity to azole drugs of the target enzyme, the cytochrome P-450 sterol 14α-demethylase, in the resistant isolate. Resistance was associated with a decreased intracellular content of drug in the resistant isolate.

Azole antifungal drugs act primarily by inhibiting the cytochrome P-450 sterol 14α-demethylase (P-450$_{14dm}$; CYP51), which is involved in the sterol 14α-demethylation reaction in ergosterol biosynthesis (34). These drugs selectively inhibit fungal P-450$_{14dm}$ by binding to the heme as a sixth ligand, with the N-1 substituent group interacting with the apoprotein (35). Azole treatment leads to the accumulation of 14α-methyl sterols like lanosterol, eburicol, obtusifoliol, 14α-methyl fecosterol and 14α-methyl-3,6-diol, at the expense of ergosterol within the cell, and this causes growth inhibition (6).

Resistance to fluconazole has become a problem with infections caused by *Candida* spp. in 10% of late-stage AIDS patients (1). Fluconazole resistance in *Candida* spp. may be acquired or intrinsic, but unlike intrinsic resistance, acquired resistance usually results in cross-resistance to other azole drugs (1, 23, 31). *Candida* spp. like *Candida krusei* are inherently resistant to fluconazole, and the widespread use of fluconazole therapy has likely contributed to an apparent increase in *C. krusei* infections (5, 42). However, *C. krusei* is generally susceptible to itraconazole (22, 28). Acquired resistance to azoles has been shown to be caused by a variety of mechanisms, including changes in the intracellular concentration of drug (9, 13, 27, 31, 38), changes in the enzymes involved in ergosterol biosynthesis (14, 41), and alterations in or the absence of the target enzyme (11, 12, 16–18, 37).

In this report we provide a biochemical characterization of itraconazole resistance in an isolate of *C. krusei* recovered from an AIDS patient. The sterol composition observed in *C. krusei* upon itraconazole treatment was similar to those observed in *Saccharomyces cerevisiae* and *Candida albicans*. The study revealed a correlation between azole resistance and decreased levels of accumulation of itraconazole within the cell.

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MATERIALS AND METHODS

Materials. Unless otherwise indicated, all chemicals (analytical grade) were purchased from Sigma, Poole, United Kingdom. Ketoconazole and itraconazole were obtained from Janssen Pharmaceuticals, Beerse, Belgium, and fluconazole was obtained from Pfizer, Sandwich, United Kingdom. [^{3}H]itraconazole was a gift from Janssen Pharmaceuticals, and [2-^{14}C]mevalonate, dibenzethylenediamine salt (specific activity, 53 mCi/mmol), was purchased from Amersham International, Bucks, United Kingdom.

Culture conditions. Strain FA0827 (an itraconazole-resistant isolate), isolated from the sputum of an AIDS patient at Monsall Hospital, Manchester, United Kingdom, and ATCC 6258 (an azole-susceptible isolate [40]) were used in the study. The cultures were maintained without selective pressure by subculturing onto Sabouraud medium (Difco) containing 2% (wt/vol) Difco Bacto Agar, and the itraconazole resistance of FA0827 was stable in repeated cultures. The MICs (the lowest concentration of drug at which no growth was observed compared with the growth of the drug-free control) of various drugs for these isolates were determined in triplicate by inoculating the cells obtained from the plates incubated at 37°C for 48 h (10^5 cells per ml) into a 60-ml Sterilin pot containing 2 ml of RPMI 1640 medium (Sigma) buffered with 0.165 M MOPS (morpholinepropanesulfonic acid; pH 7.0) and incubating the pot at 37°C for 48 h. The inhibition of growth was assessed by cell counts (hemocytometer) and determining the numbers of CFU per milliliter on Sabouraud medium (Difco) containing 2% (wt/vol) Difco Bacto Agar. MIC tests were carried out as described previously (40); a modification of the M27-P method of the National Committee for Clinical Laboratory Standards yielded identical results. Each test was repeated at least two times, and the MICs that were obtained were identical.

The same protocol was followed for analysis of the process of growth arrest, except that 0.4 μM itraconazole (the MIC for ATCC 6258) was added and the cultures were harvested at between 4 and 24 h posttreatment. Dimethyl sulfoxide (DMSO) was added to control (untreated) cultures. All drugs used in the study were dissolved in DMSO unless otherwise specified.

Sterol isolation and analysis. For sterol isolation, 100-ml cultures grown in 250-ml flasks were used. Nonsaponifiable sterols were extracted by following the method outlined by Woods (43), and gas chromatography-mass spectrometry analysis was carried out as described previously (20). Peak analysis and sterol identification were done by referring to the current literature (20, 21, 30) for relative retention times and ion fragmentation patterns.

Sterol biosynthesis in cell extracts. Preparation of cell extracts and in vitro sterol biosynthesis by using [2-^{14}C]mevalonate and cell extracts were based on the methods described previously (2, 22, 38).

Spectrophotometric analysis. The cytochrome P-450 content in the microsomal fractions (prepared by the method published previously [3]) was estimated by using the reduced carbon monoxide difference spectra obtained with a Philips PU8800 UV/VIS scanning spectrophotometer (26). Type II difference spectra were measured by adding the azole drug incrementally to the microsomal fraction in the test cuvette and an equal volume of solvent (DMSO) to the reference after recording the baseline reading from 390 to 500 nm (3, 39).

TABLE 1. Susceptibilities of *C. krusei* ATCC 6258 and FA0827 isolates to azoles and amphotericin B

Drug	MIC[a]	
	ATCC 6258	FA0827
Fluconazole	320.0	>640.0
Itraconazole	0.4	6.0
Ketoconazole	7.5	>30.0
Amphotericin B	0.1	0.1

[a] MICs are in micromolar for fluconazole, intraconazole, and ketoconazole and in milligrams per liter for amphotericin B.

Drug uptake. The intracellular content of itraconazole was investigated by following the procedure described previously (22). Cells grown to the early stationary phase were washed once and incubated at a density of 2.5×10^8 cells per ml in 0.1 M potassium phosphate buffer (pH 7.0) containing 3 μM [^{3}H]itraconazole (specific activity, 3.75 mCi/mmol) for 60 min at 37°C and 150 rpm. The cells were harvested at this time, because accumulation was shown to have reached a maximum, and they were washed three times with ice-cold saline containing unlabelled azole solution (10 μM). The cells were digested with 0.2 ml of perchloric acid and 0.5 ml of hydrogen peroxide (30%) at 70°C for 5 h, and 10 ml of scintillation solution was added before measuring the radioactivity in a Beckman LS 1801 liquid scintillation counter. The effect of sodium azide, an electron transport chain inhibitor, on [^{3}H]itraconazole uptake was examined by adding this compound (final concentration, 1 mM) to the uptake assay mixture.

RESULTS AND DISCUSSION

The MICs of various drugs for *C. krusei* FA0827 and ATCC 6258 are presented in Table 1. FA0827 was 15-fold less susceptible to itraconazole than ATCC 6258, which exhibited a susceptible response typical of other published studies on *C. krusei* (40). The itraconazole-resistant isolate was also cross-resistant to ketoconazole but not to amphotericin B, which acts by disrupting membrane function by interacting with ergosterol (25, 32). Fluconazole was less active than itraconazole and ketoconazole in inhibiting growth, with both *C. krusei* isolates exhibiting intrinsic resistance. Although comparison of the susceptibilities (MICs) of nonisogenic strains makes it difficult for one to arrive at definitive conclusions as to the cause of resistance, biochemical changes likely to cause the resistance were investigated. These included investigation of changes in sterol composition, the target enzyme, and the intracellular content of the drug.

The comparative growth rates of the two isolates in untreated cultures were investigated, and doubling times of approximately 80 min were calculated for both isolates. However, when the culture of each isolate was incubated with the MIC (0.4 μM) of itraconazole for ATCC 6258, cell growth stopped after 4 to 8 h of treatment in ATCC 6258 cultures but not in FA0827 cultures. This suggested that azole resistance is not due to alterations in growth habit, as was observed in azole-resistant *C. albicans* D10, which was also shown to be seriously defective in hyphal formation (19).

The time course changes in sterol patterns during treatment were investigated by harvesting cells at different time points after adding to growing cultures of itraconazole at the MIC (0.4 μM). Table 2 presents the percent sterol composition for ATCC 6258 at each time point during growth arrest. As was observed previously (22), this organism contains ergosterol as the principal sterol in untreated cultures. Treatment of the *C. krusei* culture with itraconazole resulted in an accumulation of 14α-methyl sterol and a decrease in ergosterol levels, reflecting P-450$_{14dm}$ inhibition. By 4 h after itraconazole exposure, growth inhibition was clear, although a substantial amount of ergosterol was still present (60.1%). The accumulation of 14α-methyl-3,6-diol continued and reached a maximum 12 h after exposure, by which time the cell numbers had reached a plateau. The other 14α-methyl sterols (obtusifoliol, 14α-methyl fecosterol, and eburicol) also accumulated immediately after treatment and reached their maximum levels within 8 to 12 h of treatment.

TABLE 2. Change in sterol composition with time in ATCC 6258 cultures after treatment with itraconazole at the MIC (0.4 μM)

Sterol	Sterol composition (%) at:							
	0 h	4 h	8 h	12 h	16 h	20 h	24 h	24 h[a]
Ergosterol	96.9	60.1	41.1	27.2	23.3	19.2	18.5	98.8
4-Methyl fecosterol	1.8	0.0	0.0	0.0	0.0	0.0	0.0	1.2
14α-Methyl fecosterol	0.0	5.8	9.0	8.7	8.1	8.0	8.8	0.0
Obtusifoliol	0.0	3.8	11.2	10.4	10.6	10.7	10.8	0.0
14α-Methyl-3,6-diol[b]	0.0	25.1	35.5	48.6	50.5	54.9	55.4	0.0
Eburicol	0.0	1.6	2.0	3.1	3.6	3.1	3.2	0.0
Unidentified sterols	1.3	3.6	1.2	3.0	3.9	4.1	3.3	0.0
Cell counts (10^5/ml)	1.0	3.0	3.5	3.8	3.8	3.9	3.9	954

[a] Control (untreated).
[b] 14α-Methyl ergosta-8,24(28)-dien-3β,6α-diol.

The sterol profile of the itraconazole-resistant isolate FA0827 and the itraconazole-susceptible isolate ATCC 6258 were investigated in the presence and absence of itraconazole (Table 3). In untreated cultures of the resistant isolate, ergosterol was the major sterol, as was the case in untreated cultures of the susceptible isolate. The levels of ergosterol in FA0827 were reduced slightly upon treatment for 24 h with itraconazole at the MIC (0.4 μM) observed for ATCC 6258. However, incubation of the FA0827 culture with itraconazole at the MIC (6.0 μM) for the isolate resulted in the accumulation of 14α-methyl sterols and a concomitant decrease in ergosterol levels consistent with P-450$_{14dm}$ inhibition.

These are the first studies on the time-dependent changes in the total sterol profile of a *Candida* spp. during growth arrest, and they revealed a close relationship between a decrease in the level of ergosterol, an increase in the level of 14α-methyl-3,6-diol, and growth arrest during itraconazole treatment. Previous studies reported that the resistance to azoles in *S. cerevisiae*, *C. albicans*, and *Ustilago maydis* was associated with a defect in the conversion of 14α-methyl fecosterol to 14α-methyl-3,6-diol by the sterol $\Delta^{5,6}$-desaturase, in which retention of

TABLE 3. Comparison of sterol profiles of ATCC 6258 and FA0827 treated for 24 h with various concentrations of itraconazole

Sterol	Sterol composition (%)				
	Untreated		Treated		
	ATCC 6258	FA0827	ATCC 6258[a]	FA0827[a]	FA0827[b]
Ergosta-tetraenol	0.0	0.0	0.0	6.3	0.0
Ergosterol	98.8	96.7	18.5	80.0	25.0
4-Methyl fecosterol	1.2	2.6	0.0	3.7	0.0
14α-Methyl fecosterol	0.0	0.0	8.8	0.0	1.5
Obtusifoliol	0.0	0.0	10.8	0.0	9.8
14α-Methyl-3,6-diol	0.0	0.0	55.4	0.0	56.6
Eburicol	0.0	0.0	3.2	0.0	7.1
Fecosterol	0.0	0.0	0.0	2.6	0.0
Ergosta-5,7-dienol	0.0	0.0	0.0	3.4	0.0
Unidentified sterols	0.0	0.7	3.3	4.0	0.0

[a] Treated with itraconazole at the MIC for ATCC 6258, 0.4 μM.
[b] Treated with itraconazole at the MIC for FA0827, 6.0 μM.

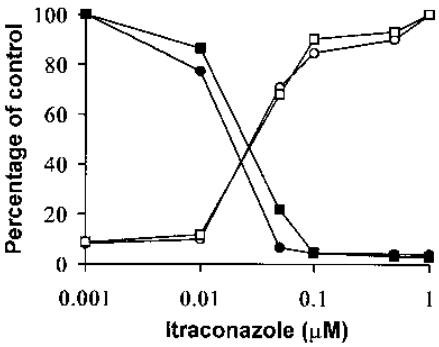

FIG. 1. Inhibition of ergosterol biosynthesis in vitro by itraconazole in ATCC 6258 (●, ergosterol; ○, eburicol) and FA0827 (■, ergosterol; □, eburicol).

14α-methyl fecosterol permitted growth (14, 17, 33, 36, 41). However ergosterol and 14α-methyl-3,6-diol are major sterols in the absence and presence, respectively, of itraconazole treatment of FA0827, indicating that no defect in sterol $\Delta^{5,6}$-desaturase or any alteration in the ergosterol biosynthetic pathway is responsible for azole resistance. However, the accumulation of 14α-methyl-3,6-diol in high quantities suggests that such a mechanism, through the alteration of $\Delta^{5,6}$-desaturase, may be found in other resistant isolates of this organism.

Inhibition of the target enzyme by azoles was studied in the two isolates to investigate whether a change in susceptibility of $P\text{-}450_{14dm}$ was responsible for itraconazole resistance. The inhibition studies were carried out by measuring the decrease in the level of incorporation of [2-^{14}C]mevalonic acid into ergosterol when cell extracts were incubated with azole drugs. Figure 1 presents the inhibition of in vitro ergosterol biosynthesis in both the isolates with various concentrations of itraconazole. The decrease in ergosterol synthesis, in the presence of itraconazole, coincided with the increase in 14α-methyl sterol levels. The concentration of itraconazole needed to inhibit 50% of ergosterol biosynthesis is provided in Table 4. The inhibition of $P\text{-}450_{14dm}$ by itraconazole (as indicated by the inhibition of ergosterol synthesis in cell extracts) occurred at a similar dose for each strain. A similar pattern of inhibition was observed with ketoconazole and fluconazole, although more fluconazole was needed for inhibition. These results suggest that the affinity of the $P\text{-}450_{14dm}$s of susceptible and resistant isolates to azoles is the same. Therefore, a mechanism of resistance based on a decreased affinity of the target enzyme to azoles, as observed for azole resistance in *Cryptococcus neoformans* and *U. maydis* (12, 18), is not plausible in this situation.

The microsomes isolated from the two isolates showed maximum absorbance at 448 nm in a carbon monoxide-reduced difference spectrum. The specific contents of P-450 in the microsomes of FA0827 and ATCC 6258 were estimated to be 30.1 ± 3.5 and 26.4 ± 5.8 pmol/mg of protein, respectively. The microsomal P-450s of the two isolates interacted with azole drugs and produced type II binding spectra which had maximum absorbances at approximately 426 nm and minimum absorbances at about 410 nm. In both isolates the interaction of P-450 with itraconazole and ketoconazole was saturated at equimolar concentrations of drug and P-450, but with fluconazole it was saturated at a 1:4 ratio of P-450 and fluconazole. These studies also indicated no alteration in either the P-450 content or the affinity of P-450 to azoles in the resistant isolate compared with that in the susceptible isolate.

TABLE 4. Half inhibitory concentrations of fluconazole, ketoconazole, and itraconazole for ergosterol biosynthesis in cell extracts of ATCC 6258 and FA0827

Drug	Half inhibitory concn (μM)	
	ATCC 6258	FA0827
Fluconazole	0.080 ± 0.010	0.097 ± 0.012
Itraconazole	0.021 ± 0.007	0.029 ± 0.005
Ketoconazole	0.023 ± 0.006	0.035 ± 0.008

The intracellular concentrations of itraconazole in FA0827 and ATCC 6258 were estimated by measuring the uptake of [^{3}H]itraconazole in order to investigate the possible involvement of reduced intracellular accumulation of drug in azole resistance. After 1 h of incubation the intracellular contents of itraconazole in ATCC 6258 and FA0827 were 1,675 ± 376 and 227 ± 28 pmol/10^9 cells, respectively, indicating approximately an eightfold difference between the two isolates in the level of accumulation of itraconazole within the cells. This may be due to a change in permeability or enhanced efflux of the antifungal agent. Resistance to azoles has also been attributed to alteration of the nonesterfied sterol/phospholipid ratio within the lipid bilayer, resulting in less permeability to drugs in AD and KB strains of *C. albicans* (10). In contrast, an increase in energy-dependent efflux has been associated with azole resistance in *Penicillium italicum*, *C. albicans*, *Candida glabrata*, and *Necteria haematococca* (4, 15, 27, 31). It is interesting in this respect that the energy-dependent efflux by multidrug resistance pumps confers resistance to several different classes of compounds in bacterial, yeast, and mammalian cells (8). Two types of membrane-bound transporter proteins (CDR1 and Ben^r) have been identified, characterized, and found to be associated with azole resistance in *C. albicans* strains isolated from AIDS patients (7, 29, 31).

In order to determine whether reduced intracellular levels of itraconazole are due to active efflux through a multidrug resistance transporter, we looked at the effect of sodium azide, a respiratory inhibitor, on itraconazole uptake. In the presence of azide the intracellular contents of itraconazole in susceptible and resistant isolates were 1,721 ± 289 and 245 ± 53 pmol/10^9 cells, respectively. The intracellular content of itraconazole was not increased by treating the resistant cells with azide, indicating that the decrease in the intracellular levels of the drug may be due to a decrease in permeability to the drug. However, we cannot rule out the presence of a multidrug resistance transporter energized by membrane potential and proton motive force (24) and its involvement in azole resistance in *C. krusei.*

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