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Comparison of D0870, a New Triazole Antifungal Agent, to Fluconazole for Inhibition of *Candida albicans* Cytochrome P-450 by Using In Vitro Assays

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D0870 was 12 to 15 times more active than fluconazole in experiments to determine the MIC for growth arrest for two isolates of *Candida albicans*. A biochemical comparison of in vitro sterol biosynthesis in cell extracts showed only a twofold superiority of D0870 over fluconazole. A large differentiation (10-fold) in 50% saturating concentrations obtained by examining the binding of the azoles to microsomal P-450 was observed in a type II binding spectrophotometric assay, possibly reflecting the differential affinity for more than one P-450 enzyme. Additional mechanisms besides affinity for the target enzyme sterol 14α-demethylase, such as differential intracellular accumulation of drug, may contribute to the differences in antifungal activity.

Infections caused by opportunistic fungal pathogens, particularly *Candida albicans*, have increased dramatically in the past 20 years because of increasing numbers of organ transplantations, increasing use of chemotherapy for cancer treatment, intensive care medicine, and increasing numbers of patients with human immunodeficiency virus infection (6, 18, 27). Fluconazole, a fungistatic triazole drug (Fig. 1), has been used with considerable success in the treatment of both mucosal and invasive candidoses (14). However, the number of patients, particularly AIDS patients, failing treatment has increased because of infection with fluconazole-resistant *Candida* species (2, 29). Resistance is at least partly related to the long-term use of fluconazole, but it is also related to declining immune status (2). D0870, a new triazole antifungal agent developed by Zeneca (Macclesfield, United Kingdom), is an enantiomer of ICI 195,739 (Fig. 1). The superiority of this drug over other azole drugs has been observed for a wide range of pathogenic yeasts including *Candida* and *Cryptococcus* spp., although its activity against *Aspergillus* spp. is marginal (9, 25, 28, 35, 38).

The differences in both the potencies and selectivities between azole antifungal drugs are due to variations in the concentrations of the drugs in serum, the levels of intracellular accumulation of the drugs, and the differential affinities of the drugs for the target enzyme. Affinity with the target enzyme, cytochrome P-450-dependent sterol 14α-demethylase ($P\text{-}450_{dm}$) (34), can be investigated by using the ability of carbon monoxide to displace azole drugs from reduced $P\text{-}450_{dm}$ (8, 33, 34), by measuring the ability of azole antifungal agents to induce type II difference spectra on binding to $P\text{-}450_{dm}$, or by measuring the inhibitory effects of azole drugs on ergosterol biosynthesis in cell extracts or in reconstituted systems with purified $P\text{-}450_{dm}$ (16).

In the study described in the present report, the inhibitory effect of D0870 on the in vitro cell growth and ergosterol biosynthesis in cell extracts of two clinical isolates of *C. albicans* and the interaction of D0870 with microsomal cytochrome P-450 were studied and compared with the effect of fluconazole. The results obtained by different methods were compared and are discussed in relation to the toxicity of D0870 to *C. albicans*.

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MATERIALS AND METHODS

Strains. *C. albicans* Ca505 and Ca033 were obtained from AIDS patients (35).

Chemicals. Unless specified otherwise, all chemicals were obtained from Sigma Chemical Company, Poole, United Kingdom. Fluconazole was purchased from Pfizer (Sandwich, United Kingdom), D0870 was a gift from Zeneca, and [2-^{14}C]mevalonate, dibenzethylenediamine salt (specific activity, 53 mCi/mmol), was obtained from Amersham International (Bucks, United Kingdom). Fluconazole at 1 μM corresponds to 0.31 μg of fluconazole per ml, and D0870 at 1 μM corresponds to 0.54 μg of D0870 per ml.

Growth inhibition studies. Stationary-phase cells were obtained from plate cultures incubated at 37°C on Sabouraud medium (Difco) with 2% (wt/vol) Difco Bacto agar and were inoculated in 2 ml of RPMI 1640 medium (Sigma) contained in a 60-ml Sterilin container at 10,000 cells per ml. Treatment with various doses of antifungal compound (dissolved in dimethyl sulfoxide [DMSO]) occurred over 2 days at 37°C and 150 rpm, and growth was assessed by measuring cell counts and determining the numbers of CFU per milliliter on YEPD, consisting of 2% (wt/vol) glucose, 2% (wt/vol) Difco Bacto peptone, 1% (wt/vol) Difco yeast extract, and 2% (wt/vol) Difco Bacto agar. MIC tests were performed as described previously; a modification of the M27-P method of the National Committee for Clinical Laboratory Standards yielded identical results with proposed quality control isolates (35). Each test was repeated at least three times, and MICs and half inhibitory concentrations (IC_{50}s) were constant.

Identification of sterols by gas chromatography-mass spectrometry. Samples for gas chromatography-mass spectrometry were prepared from 100-ml cultures obtained by incubating cells in the presence or absence of drug at the MICs for 24 h in RPMI 1640 medium. The cell pellet was saponified in 15% (wt/vol) KOH in 90% (vol/vol) ethanol at 80°C for 1 h. Nonsaponifiable lipids (sterols and sterol precursors) were extracted three times with 5 ml of hexane and dried under nitrogen. Following silylation for 1 h at 60°C with BSTFA (20 μl) in 100 μl of toluene, sterols were analyzed by gas chromatography-mass spectrometry (VG 12-250; VG BIOTECH) by using split injections with a split ratio of 20:1. Sterol identification was by reference to relative retention times and mass spectra, as reported previously (17, 30).

Cell-free ergosterol biosynthesis assay. Fluconazole and D0870 inhibition of P-450 was investigated by assessing the cell-free biosynthesis of ergosterol (sterol 14α-demethylation activity) by methods similar to those reported previously (3, 5, 23). After growth of 1 liter of culture in 2-liter flasks at 150 rpm and 37°C to the late logarithmic phase, cells were homogenized with a Braun disintegrator (Braun GmbH, Mesungen, Germany) operating at 4,000 rpm with four 30-s bursts with liquid carbon dioxide cooling. A total of 20 g of glass beads (diameter, 0.45 to 0.5 mm) were mixed with the cells and made up to 50 ml with 20% (wt/vol) glycerol–100 mM phosphate buffer (pH 7.4). Cell extracts were obtained

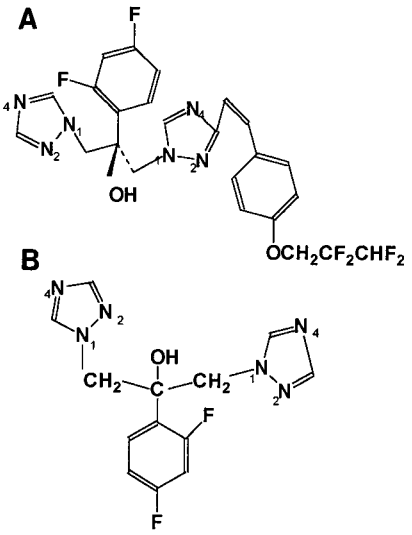

FIG. 1. Structures of D0870 (A) and fluconazole (B).

following centrifugation at 1,500 × *g*, and the protein concentration was estimated by the bicinchoninic acid method (Sigma). The reaction mixture consisted of cell extract (924 μl/ml; protein concentration, 10 to 15 mg/ml), cofactor solution (50 μl; containing 1 μmol of NADP, 1 μmol of NADPH, 1 μmol of NAD, 3 μmol of glucose-6-phosphate, 5 μmol of ATP, and 3 μmol of reduced glutathione in distilled water), divalent cation solution (10 μl of 0.5 M $MgCl_2$ and 5 μl of 0.4 M $MnCl_2$), and a solution of an azole antifungal compound dissolved in DMSO (1 μl)–[2-^{14}C]mevalonate (10 μl; 0.25 μCi) and was adjusted to pH 7.4 (by the addition of 10 M KOH). The mixture was incubated at 37°C for 2 h with shaking (110 rpm), after which the reaction was stopped by adding 1 ml of freshly prepared saponification reagent (15% [wt/vol] KOH in 90% [vol/vol] ethanol) and heated at 80°C for 1 h for saponification. Nonsaponifiable lipids (sterols and sterol precursors) were extracted twice with 3 ml of petroleum ether (boiling point, 40 to 60°C) and dried under nitrogen. The nonsaponifiable lipid was applied to silica gel thin-layer chromatography plates (ART 573; Merck) and was developed with toluene-diethyl ether (9:1; vol/vol). Radioactive sterols were located by autoradiography and were excised for scintillation counting. The production of ergosterol was assessed for inhibition as described previously (3, 5, 23). Experiments were performed in triplicate, and IC_{50}s for the inhibition of ergosterol biosynthesis were calculated.

Isolation of microsomal cytochrome P-450. Microsomal fraction isolation was carried out as described previously (8, 19). The cells harvested from the late logarithmic phase were washed once and were resuspended in 50 mM citrate-phosphate buffer (pH 5.8) containing 1.2 M sorbitol and 20 mM 2-mercaptoethanol at 2×10^9 cells per ml, and the mixture was digested with Novozyme (2 mg/ml) at 30°C for 30 min with gentle agitation. All subsequent steps were carried out at 4°C. Spheroplasts were harvested by centrifugation at 5,000 × *g* for 5 min; washed three times with ice-cold 100 mM phosphate buffer (pH 7.5) containing 0.1 mM EDTA, 0.1 mM reduced glutathione, and 20% (vol/vol) glycerol (buffer A); and then resuspended in buffer A without glycerol at 2×10^9 cells per ml to break the spheroplasts by osmotic shock. The mixture was then homogenized with a Potter-Elvehjem glass homogenizer. The homogenate was left on ice for 20 min and was centrifuged at 5,000 × *g* for 5 min to remove the cell debris. The supernatant was centrifuged twice at 12,000 × *g* for 20 min to remove the mitochondria and then at 105,000 × *g* for 90 min to pellet the microsomal fraction. The microsomal pellet was resuspended in buffer A with a Potter-Elvehjem glass homogenizer, and the mixture was stored at −80°C until further use. The protein concentration was estimated by the bicinchoninic acid method (Sigma).

Cytochrome P-450 content estimation. The cytochrome P-450 concentration was measured as described previously (26). Microsomal suspensions reduced with sodium dithionite were transferred to two quartz cuvettes, and the baseline was recorded with a Philips PU8800 UV/VIS scanning spectrophotometer. The contents in the sample cuvette were bubbled with carbon monoxide for 45 s at a rate of one bubble per second, and the difference spectra were recorded.

Type II binding spectra. Microsomal suspensions (100 nM) were transferred into each of two quartz cuvettes, and a baseline was recorded from 390 to 500 nm. Difference spectra were then recorded after incremental additions of the azole antifungal compound (dissolved in DMSO) to the test cuvette and an equal volume of solvent to the reference cuvette. The final concentration of DMSO did not exceed 1% (vol/vol) (4, 16).

Carbon monoxide displacement spectra. Carbon monoxide displacement spectra studies were carried out as described previously (14). A total of 100 pmol of microsomal P-450 was incubated on ice with 5 μM an azole (1% DMSO in the control experiment) compound for 2 min, and then the reduced CO difference spectrum was recorded as described above at 5-min intervals for 30 min after the start of CO treatment.

TABLE 1. MICs and IC_{50}s of fluconazole and D0870 for two *C. albicans* isolates

Drug and *C. albicans* isolate	IC_{50} (μM)	MIC (μM)
D0870		
Ca033	0.5	1.8
Ca505	0.3	0.9
Fluconazole		
Ca033	7.6	25.6
Ca505	3.8	12.8

RESULTS

Toxicity assay. The inhibitory effect of D0870 was studied by using two clinical isolates of *C. albicans*, isolates Ca033 and Ca505, and was compared with the inhibitory effect of fluconazole (Table 1). The MIC and IC_{50} of fluconazole were 12- to 15-fold higher than those of D0870 for the inhibition of in vitro cell growth. Analysis of the two isolates showed ergosterol as the major sterol and smaller quantities of intermediates of the pathway. Treatment of both isolates with D0870 for 24 h resulted in the reduction of ergosterol to nondetectable levels and the accumulation of 14α-methyl-3,6-diol to high levels (Table 2). This shift in sterol composition also occurred in fluconazole-treated isolates.

Cell-free sterol 14α-demethylation assay. The susceptibility of $P\text{-}450_{dm}$ to D0870 and fluconazole was assessed by studying the inhibition of ergosterol biosynthesis in cell extracts of Ca033 and Ca505 (Fig. 2). D0870 inhibited the incorporation of [2-^{14}C]mevalonate into ergosterol at concentrations lower than those of fluconazole. The IC_{50}s (the concentrations which inhibit the incorporation of mevalonate into ergosterol by 50%) of D0870 were approximately half those of fluconazole (Table 3).

TABLE 2. Relative sterol compositions of *C. albicans* isolates after 24 h of incubation with or without azole drug at the MIC

Sterol	% of total sterols[a]					
	Ca033			Ca505		
	Cont[b]	Flu[c]	D[d]	Cont	Flu[e]	D[f]
Ergosta-tetraenol	ND[g]	ND	ND	3.5	ND	ND
Ergosterol	65.1	ND	ND	64.4	ND	ND
Fecosterol	2.4	ND	ND	5.7	ND	ND
Ergosta-5,7-dienol	ND	ND	ND	10.7	ND	ND
Episterol	8.0	ND	ND	6.5	ND	ND
Obtusifoliol	18.0	15.9	9.7	6.1	16.3	12.1
Eburicol	ND	15.0	15.2	2.0	17.6	19.1
14α-Methyl-3,6 diol[h]	ND	60.0	56.4	ND	66.1	64.6
14α-Methyl fecosterol	ND	9.1	9.4	ND	ND	4.2
Unidentified sterols	6.5	ND	9.3	2.1	ND	ND

[a] Sterol proportions varied by less than 10% in three experiments.
[b] Cont, control (no drug).
[c] Flu, fluconazole at 25.6 μM.
[d] D, D0870 at 1.8 μM.
[e] Flu, fluconazole at 12.8 μM.
[f] D, D0870 at 0.9 μM.
[g] ND, not detected.
[h] 14α-Methylergosta-8,24(28)-diene-3β,6α-diol.

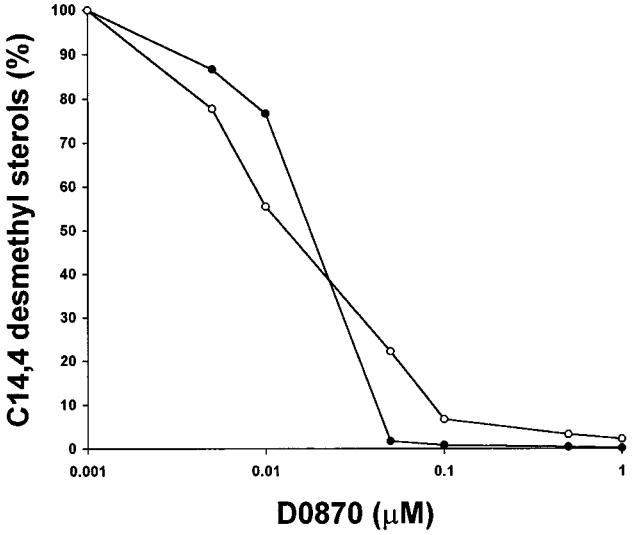

FIG. 2. Inhibition of incorporation of [2-^{14}C]mevalonate into ergosterol in cell-free bioassays of Ca033 (●) and Ca505 (○) isolates of *C. albicans* by D0870.

Spectrophotometric studies. Microsomes isolated by mechanical cell disruption with glass beads did not show reduced CO difference spectrum maxima at 450 nm because of cytochrome oxidase contamination (data not shown), which has negative absorbance in the region of the Soret maximum for P-450. In order to eliminate this problem, microsomes were isolated by gently lysing the spheroplasts produced with Novozyme. The specific contents of the microsomal cytochrome P-450 of Ca033 and Ca505 were 50 ± 8 and 44 ± 4 pmol/mg of protein, respectively. The type II difference spectra of the microsomal cytochrome P-450 of the Ca033 and Ca505 *C. albicans* isolates were recorded for D0870 and fluconazole. The type II spectral changes induced by D0870 and fluconazole were characterized by an absorbance maximum at 428 to 430 nm and an absorbance minimum at 408 to 412 nm. The magnitude of spectral change was greater with D0870 (0.1 μM) than with fluconazole (1.0 μM) and was linearly dependent on the concentrations of azole added to the microsomal P-450. For D0870 the saturation response in the type II spectra was attained at almost equimolar concentrations of P-450 (0.1 μM) and antifungal compound for both isolates, isolates Ca033 and Ca505, of *C. albicans*. For fluconazole, saturation of the response was found at 1:10 molar concentrations of P-450 and drug, respectively (Fig. 3). D0870 exhibited lower IC_{50}s (half saturation response) compared with the IC_{50}s of fluconazole (Table 3).

TABLE 3. IC_{50}s of D0870 and fluconazole for incorporation of [2-^{14}C]mevolanate into ergosterol in cell-free bioassays and IC_{50}s of D0870 and fluconazole which induced response in type II difference spectrum in microsomal cytochrome P-450 of *C. albicans* isolates

Drug and *C. albicans* isolate	IC_{50} (nM)	
	In vitro sterol biosynthesis	Type II binding spectra
D0870		
Ca033	27.5 ± 5.2	7.8 ± 0.6
Ca505	17.5 ± 2.3	8.2 ± 0.4
Fluconazole		
Ca033	55.0 ± 4.2	100.7 ± 7.8
Ca505	42.5 ± 7.9	97.5 ± 6.9

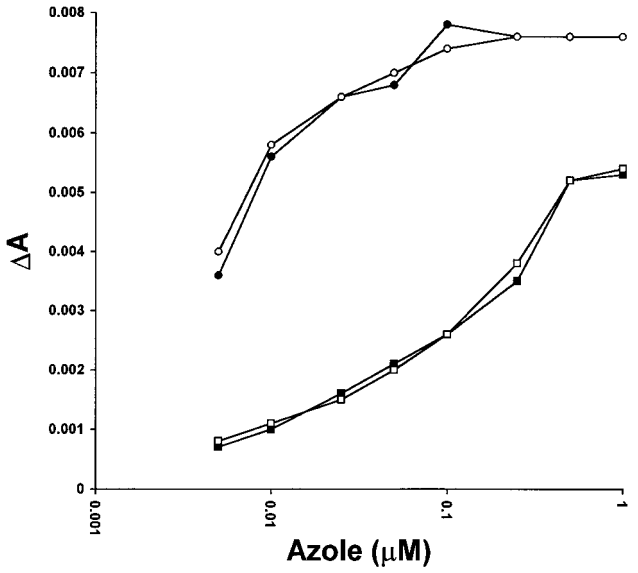

FIG. 3. Relation between D0870-fluconazole concentrations and the magnitude of type II difference spectra on binding to the microsomal cytochrome P-450 (0.1 μM) of the Ca033 (D0870 [●] and fluconazole [■]) and Ca505 (D0870 [○] and fluconazole [□]) isolates of *C. albicans*. The magnitude of the type II difference spectra (ΔA) is the difference between its maximum (428 to 430 nm) and the minimum (405 to 410 nm).

The relative affinities of D0870 and fluconazole for the ferrous P-450 were studied by determining their inhibitory effects on CO–P-450 complex formation. Both of the azole drugs were able to interfere with the binding of CO, but to significantly different degrees (Fig. 4). D0870 was a more potent inhibitor of CO–P-450 complex formation than fluconazole for both

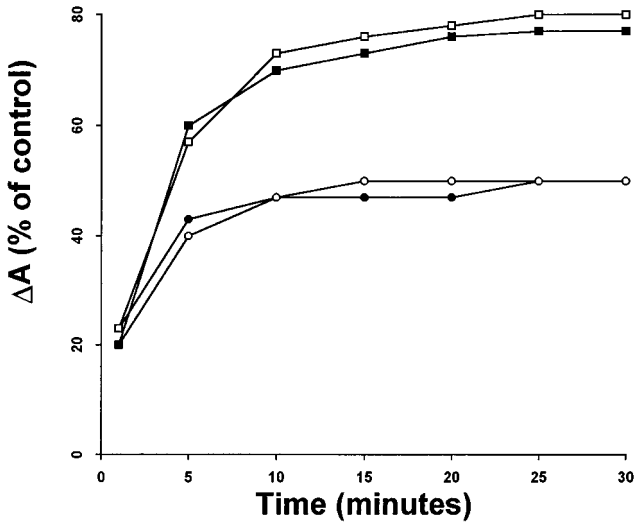

FIG. 4. Carbon monoxide displacement of D0870 and fluconazole from the microsomal cytochrome P-450 of the Ca033 (D0870 [●] and fluconazole [■]) and Ca505 (D0870 [○] and fluconazole [□]) isolates of *C. albicans*. ΔA is the difference in absorbance between the maximum at 448 nm and the minimum at 490 nm of the reduced CO difference spectrum. The change in absorbance during incubation at room temperature after CO treatment is given as a percentage of the value for the controls.

isolates, isolates Ca033 and Ca505, of *C. albicans*. After 30 min of incubation, the average displacement efficiencies of D0870 and fluconazole in both isolates were 50 and 80%, respectively. Specifically, the CO displacement efficiency of D0870 (5 μM) from microsomal cytochromes P-450 (0.1 μM) of *C. albicans* Ca033 and Ca505 30 min. after CO treatment was 50% ± 3% and 50% ± 5%, respectively. That of fluconazole (5 μM) was 77% ± 9% and 80% ± 6%, respectively.

DISCUSSION

The search for novel, systemically active antifungal agents is now in progress all over the world, and the triazole agents fluconazole and itraconazole have been developed as useful therapeutic agents over the last decade. However, azole, and particularly fluconazole, resistance in *C. albicans* has emerged as a major problem in AIDS patients and occasionally in patients in intensive care units (2, 11, 15, 21, 29, 36). Furthermore, many species of *Candida* are inherently tolerant of fluconazole (1, 7, 24, 37). Under these circumstances, more potent antifungal agents which are safe and efficacious are urgently needed (10).

In the present study the in vitro activity of D0870 was evaluated against two clinical isolates of *C. albicans* and was compared with that of fluconazole. Comparing MICs and IC_{50}s between D0870 and fluconazole, we found that D0870 was much more active than fluconazole for the inhibition of growth of *C. albicans* isolates in vitro. It is of interest that the two methods yielded results in direct proportion to each other for both drugs. In both isolates of *C. albicans* tested, isolates Ca033 and Ca505, ergosterol became reduced to undetectable levels in the presence of D0870 or fluconazole after treatment at the MICs of both drugs. This suggests that the mode action of D0870 is the same as that of fluconazole.

The correlation between the toxicities of the compounds against fungi and their ability to inhibit cell-free sterol 14α-demethylation was examined, but the large difference in toxicity between fluconazole and D0870 could not be explained by the relatively small difference (twofold) in the inhibitory effects between these two drugs. Thus, the differential toxicities of D0870 and fluconazole are determined not only by their differential affinities for the target enzyme, $P\text{-}450_{dm}$, but also by additional mechanisms such as uptake or efflux (13). In the present study fluconazole and D0870 induced type II difference spectra with microsomal cytochromes P-450 of two isolates of *C. albicans*. This indicated that the N-4 of the triazole ring of D0870 and fluconazole was bound to the heme of P-450 as a sixth ligand (31). Comparison of the type II difference spectra of fluconazole and D0870 showed a difference in the magnitude of the spectrum obtained at saturating concentrations of the drugs. For fluconazole at saturation levels, the size of the type II spectrum was about 60% that obtained with D0870. The precise reason for this difference is not known.

The CO displacement test has previously been reported as being suitable for assessing the in vitro antifungal activities of azole antifungal drugs (8, 32, 33), but more recently, this has been challenged (4, 12, 17, 22). D0870 and fluconazole caused inhibition of CO–P-450 complex formation which did not correlate quantitatively with their toxicities but which did reflect the differences observed in cell-free sterol 14α-demethylation inhibition. It was therefore concluded that the CO displacement tests could be used for structure-activity studies. This observation is supportive of previous studies which showed a correlation between inhibition of sterol 14α-demethylation by various antifungal drugs and their ability to impair the CO–$P\text{-}450_{dm}$ complex formation (32, 33).

The concentration of fluconazole which induced a half saturation response of type II difference spectra (IC_{50}) was 12- to 15-fold higher than that of D0870, and the difference in the IC_{50}s for type II spectra correlated with their toxicities to *C. albicans* isolates in in vitro cell growth assays. However, it is very unlikely that the 12- to 15-fold differences in the half saturation concentrations for type II binding spectra accounts for the differences in the MICs or IC_{50}s for cell growth, because a correlation was not observed in inhibition of cell-free sterol 14α-demethylation or in CO displacement tests. P-450 is located in the cytosol, although it is anchored to the membrane by an N-terminal leader sequence. Consequently, differential solubilities may not cause the differences observed between the compounds. However, the active-site access channel may be held in close association with the membrane to allow entry of the relatively insoluble sterol substrates. This could favor access by compounds which partition into the membrane, but differences in the direct measurement of enzyme inhibition in cell-free sterol biosynthesis would be expected to be detectable. An alternative explanation is that the differences in the type II spectra produced during the addition of an azole could reflect differences in binding to the total P-450 complement. Gene disruption of $P\text{-}450_{dm}$ in *Saccharomyces cerevisiae* has demonstrated the presence of an additional P-450 enzyme(s) with sterol Δ^{22}-desaturase activity (20).

In conclusion, the large difference in toxicity between D0870 and fluconazole could not be explained by relatively small differences in the CO displacement and inhibition of sterol 14α-demethylation. The difference in toxicity observed might be due to variations in the intracellular level of accumulation of D0870 and fluconazole. However, studies on measuring the accumulation of drugs intracellularly were not carried out because of the unavailability of radiolabelled drugs.

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