Amino acid variations of cytochrome P-450 lanosterol 14α-demethylase (CYP51A1) from fluconazole-resistant clinical isolates of *Candida albicans*

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Summary

We studied six clinical isolates of *Candida albicans.* All six isolates showed high level resistance to fluconazole (minimum inhibitory concentrations 64 µg/ml) with varying degrees of cross-resistance to other azoles but not to amphotericin B. Neither higher dosage nor upregulation of the gene encoding the cytochrome P-450 lanosterol 14 α-demethylase (CYP51A1 or P-450LDM) was responsible for fluconazole resistance. The resistant and the susceptible isolates accumulated similar amounts of azoles. To examine whether resistance to fluconazole in these clinical isolates of *C. albicans* is mediated by an altered target of azole action, we cloned the structural gene encoding P-450LDM from the fluconazole-resistant isolates. The amino acid sequences of the P-450LDMs from the isolates were deduced from the gene sequences and compared to the P-450LDM sequence of the fluconazole-susceptible *C. albicans* B311. The enzymes from the clinical isolates showed 2 to 7 amino acid variations scattered across the molecules encompassing 10 different loci. One-half of the amino acid changes obtained were conserved substitutions (E116D, K143R, E266D, D278E, R287K) compared to the susceptible strain. Non-conserved substitutions were T128K, R267H, S405F, G450E and G464S, three of which are in and around the heme-binding region of the molecule. R287K is the only amino acid change that was found in all six clinical isolates. One or more of these mutational alterations may lead to the expression of an azole-resistant enzyme.

Key words *C. albicans*, Fluconazole, Resistance, Demethylase

Variaciones en los aminoácidos de la lanosterol 14α-demetilasa del citocromo P-450 (CYP51A1) en aislamientos clínicos de *Candida albicans* resistentes al fluconazol

Resumen

Estudiamos seis aislamientos clínicos de *Candida albicans* que presentaban resistencia importante al fluconazol (concentraciones mínimas inhibitorias 64 µg/ml) on diferente grado de resistencia cruzada a otros azoles pero no a la anfotericina B. La resistencia al fluconazol no era debida a una mayor cantidad ni a la sobreregulación del gen que codifica la 14α-demetilasa del citocromo P-450. Tanto los aislamientos resistentes como los sensibles acumularon cantidades similares de azoles. Para examinar si la resistencia al fluconazol de estos asilamientos clínicos de *C. albicans* está mediada por una alteración en la diana del azol, clonamos el gen estructural que codifica la P-450LDM en los aislamientos resistentes al fluconazol. Las secuencias de aminoácidos de las P-450LDMs de los aislamientos se dedujeron a partir de las secuencias génicas y se compararon con la secuencia de la P-450LDM de la cepa de *C. albicans* B311, sensible al fluconazol. Los enzimas de los aislamientos clínicos presentaron de dos a siete variaciones en aminoácidos distribuídos a través de moléculas que impli-

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Aceptado para publicación el 28 de julio de 1999

1130-1406/99/5.00 Euros

can a 10 loci diferentes. La mitad de los cambios obtenidos en los aminoácidos eran sustituciones conservadas (E116D, K143R, E266D, D278E, R287K) respecto a la cepa sensible. Las sustituciones no conservadas fueron T128K, R267H, S405F, G450E y G464S, tres de las cuales están in and around the heme-binding region de la molécula. R287K fue el único cambio en aminoácidos encontrado en los seis aislamientos clínicos. Una o varias de estas alteraciones mutacionales puede conllevar la expresión de un enzima resistente a los azoles.

Palabras clave *C. albicans*, Fluconazol, Resistencia, Demetilasa

Candida albicans is a dimorphic yeast that causes a wide spectrum of diseases in man ranging from mild superficial infection of the skin and mucosa to severe disseminated candidiasis in immunocompromised individuals. Fluconazole (FLZ) is a commonly used antifungal agent, which possesses several desirable pharmacological characteristics (e.g., soluble in aqueous solvents, high serum concentration, low toxicity and relatively long serum half-life). Fluconazole inhibits ergosterol biosynthesis in *C. albicans* by inhibiting the enzyme cytochrome P-450 lanosterol 14 α-demethylase (P-450LDM). This enzyme named P-450LDM, Erg11 or CYP51A1 is responsible for the demethylation of lanosterol an essential intermediate step in the synthesis of ergosterol. Although FLZ has been used successfully to treat candidiasis in immunocompromised patients, including patients with AIDS, its frequent and long term usage has resulted in the selection of resistant isolates of *C. albicans* from patients with AIDS [6,14,27]. Recent reports describing the emergence of FLZ-resistant isolates has been considered responsible for the increasing incidence of clinically refractory mucosal candidiasis in advanced AIDS patients. Thus, the continued effective use of FLZ for the management of mucosal candidiasis in AIDS patients has been severely compromised by the emergence of azole-resistance in *C. albicans*.

Three mechanisms are known to be present in *C. albicans* producing fluconazole resistance. Firstly, the decreased accumulation of the drug in the cell, which occurs via the alteration of the permeability of azoles into the cell [8] or by the expulsion of the drug taken up by the cell at the expense of energy [15,20-22,29]. The net result of these processes is the subsequent decrease in intracellular concentration of the drug, to the extent that it has no effect on cellular function [25,26]. The second known mechanism of azole resistance in *C. albicans* is the increased synthesis of P-450LDM, the target of azole action, either by gene amplification or due to upregulation of synthesis [29]. The end result is the production of increased amounts of P-450LDM, hence more drug is required to inhibit the activity of the enzyme. The third described mechanism of resistance to azoles, in particular to FLZ, is the alteration of the target enzyme (P-450LDM) by mutational changes [12,23,24,28]. On occasion, these changes on the target protein lead to structural alteration of the protein such that the enzyme is refractory to the inhibitory action of the drug, and thus the organism is resistant to the azole. An understanding of the mechanism(s) of resistance to azoles, and in particular to FLZ, at the molecular level is essential. This information is valuable in the development of newer azoles that are effective against FLZ-resistant pathogenic yeasts, and for the design of new and improved antifungals that inhibit the sterol biosynthesis in fungi.

In this study, we have investigated one possible molecular mechanism which is responsible for the azole resistance trait found in several clinical isolates of *C. albicans* obtained from patients treated at the Detroit Medical Center with AIDS and clinically resistant mucosal candidiasis.

MATERIALS AND METHODS

Organisms and culture conditions. The organisms evaluated include seven *C. albicans* isolates. Six of the isolates are FLZ-resistant clinical isolates (DC, F01A, PL01A, RC, ZW02 and ZS04) of *C. albicans.* The isolates were recovered from six different patients with advanced AIDS and FLZ-refractory oral candidiasis. In addition, as a control, the well described FLZ-susceptible *C. albicans* laboratory strain B311 (ATCC 32354) was also used in this study. The yeast cultures were grown in peptone yeast extract glucose (PYG: peptone 1 g, yeast extract 1 g, glucose 3 g, per liter of distilled water) medium for 24 h at 30°C on a shaker with constant agitation (150 rpm). The cells were harvested by centrifugation, washed with ice-cold TE (Tris 10 mM, pH 8.0; EDTA 1 mM) buffer and the pellet was stored at -20°C before use.

Antifungal agents. Amphotericin B, FLZ, ketoconazole, itraconazole, clotrimazole, miconazole and flucytosine were obtained from their respective manufacturers. Stock solutions (1-2 mg/ml) were prepared either in sterile distilled water (FLZ and flucytosine) or in dimethylsulfoxide (clotrimazole, ketoconazole, miconazole, itraconazole and amphotericin B) and stored at -70°C. A new stock solution was used each day the assays were performed. The final concentrations ranged from 0.08-80 µg/ml for FLZ, 0.01-6.25 µg/ml for itraconazole, ketoconazole, clotrimazole, miconazole and amphotericin B, and 0.04-20 µg/ml for flucytosine.

Determination of minimum inhibitory concentration. The minimum inhibitory concentrations (MICs) of all antifungal agents for the various *C. albicans* isolates were determined by the broth microdilution technique according to the recommendations of the National Committee for Clinical Laboratory Standards [5, 13]. The end-points were determined spectrophotometrically. The MICs of the azoles and flucytosine were defined as the lowest concentrations that inhibited 80% of visible growth compared with the growth control. The MICs of amphotericin B were defined as the lowest concentrations that inhibited 100% of the visible growth.

Molecular cloning and characterization of the gene encoding P-450LDM. Genomic DNA from various *C. albicans* isolates was prepared using a modified procedure from that was described previously [3]. Briefly, cells were harvested by centrifugation, washed and resuspended (1 g wet weight/5 ml) in protoplasting medium

(0.15 M phosphate buffer, pH 7.5, 0.9 M sorbitol) containing 1 mM dithiothreitol and 100 units of lyticase (Sigma, USA). Afterwards, the cells were incubated at 37°C for 2 h with occasional agitation. The protoplasts were lysed with the addition of 6 ml of TE (50 mM Tris, pH 8.0; 50 mM EDTA) buffer and 1 ml of 10% sodium dodecyl sulfate. The mixture was incubated with gentle shaking for 1 hr at 37°C and the lysate was centrifuged at 20,000 x g for 30 min at 4°C. The clear supernatant was collected and the genomic DNA was precipitated with ethanol. The DNA was collected by centrifugation, dissolved in 5 ml of TE (50 mM Tris, pH 8.0; 50 mM EDTA) and purified using Qiagen Column (Qiagen, usa) according to the recommendation of the supplier [16]. The purified DNA was precipitated with 0.75 volume of isopropanol and the quality of the DNA was evaluated by agarose gel electrophoresis.

The complete structural gene encoding the P-450LDM from the clinical *C. albicans* isolates was amplified using the oligodeoxynucleotides: 5'-ATGGCTATTGTTGAAACTGTCATTG-3'and 5'-AAACATACAAGTTTCTCTTTTTTCCC-3' as primers by polymerase chain reaction (PCR). The resulting 1584-bp DNA fragment was ligated to the pGEM-T vector (Promega Corp., USA) and introduced in *E. coli* by electroporation. The recombinant plasmid propagated in *E. coli* was isolated and the complete nucleotide sequence of the cloned P-450LDM gene was determined by dideoxy chain termination reaction [19] using Sequenase Version 2.0 (United States Biochemical, USA). The coding and the non-coding strands of the P-450LDM gene from at least two of the recombinant clones representing each *C. albicans* isolate were sequenced completely to avoid sequencing error as well as to eliminate nucleotide changes introduced by PCR. The DNA and protein sequence analyses, and the prediction of secondary structure of the protein were performed by the PC Gene DNA and Protein Sequence Analysis Program (Intelligenetics, USA).

Itraconazole uptake assay. To measure the intracellular accumulation of itraconazole in *C. albicans*, 10 ml of cell suspension (1 x 10^7 cells per ml) was incubated with 10 µM itraconazole containing 167 nCi per ml of ^{3}H-itraconazole (13.5 Gbg/ml) in growth medium at 30°C. One-tenth ml aliquots were removed at various time intervals, collected on millipore filters (HAWP 0.22 µm) and washed two times rapidly with ice-cold growth medium (10 ml each). The filters containing the cells were dried using a heat lamp and the radioactivity associated with the filters were determined by scintillation counting.

Genome blot analysis. Genomic DNA (10 µg each) was digested with the restriction enzymes *Eco*R1, *Hind*III, *Pst*1, *Sty*1 and *Xho*1 individually. The restricted DNA fragments were separated by agarose gel electrophoresis and transferred to a nylon membrane by Southern blot. The nylon membrane was hybridized at high stringency conditions (42°C, 50% formamide) with 32P-labelled DNA probe derived from the complete P450LDM structural gene of *C. albicans* B311, washed at 65°C and exposed to Kodak XAR-5 X-ray film for autoradiography.

RESULTS

Susceptibility studies. The MICs of the various azoles and amphotericin B for the FLZ-resistant clinical isolates and the FLZ-susceptible laboratory strain B311 are shown in Table 1. The FLZ MIC values for the clinical isolates were at least 64 fold higher than that obtained for *C. albicans* B311. In addition, the clinical isolates demonstrated varying degrees of cross-resistance to other members of the azole family of antifungals. For instance, only 4 of the 6 isolates (DC, PL01A, RC and ZS04) with higher MIC values for FLZ showed a significant rise in the MIC of MCZ. Similarly, three of the six isolates (DC, ZS04 and RC) showed a significant increase in the clotrimazole MICs (78-156 fold), whereas the remaining three isolates had MIC values similar to the control strain of *C. albicans* B311 (F01A), or slightly higher than that of B311 (ZW02 and PL01A). On the other hand, only two of the isolates (DC and ZSO4) had any significant increase in the MIC to itraconazole (0.5µg/ml) when compared to the B311 strain (0.125 µg/ml). There was no significant increase in the MIC of ketoconazole detected for any of the clinical isolates studied. While the various *Candida* isolates demonstrated varying degrees of *in vitro* azole cross-resistance, none of the isolates showed decreased susceptibility to either amphotericin B or flucytosine. Thus, revealing the lack of cross-resistance between azoles and amphotericin B and flucytosine in these isolates. In addition, these results suggest that reduced susceptibility to one azole (e.g. FLZ) among *C. albicans* does not necessarily mean that they are equally less susceptible to other azoles.

Table 1. *In vitro* susceptibility of fluconazole-resistant clinical isolates of *C. albicans* and the laboratory strain B311 to various antifungals.

C. albicans isolates	Minimum Inhibitory Concentration (µg per ml)						
	FLZ	MCZ	ITZ	CLZ	KTZ	AMB	5FC
B311	0.5	0.5	0.125	0.008	0.125	0.4	0.16
DC	>64	>4	0.50	4	0.03	0.4	0.16
F01A	>64	0.25	0.016	0.016	0.016	0.4	0.39
PL01A	32	4	0.06	0.50	0.016	0.2	0.16
RC	>64	>4	0.06	4	0.03	0.2	0.16
ZW02	>64	0.25	0.06	0.50	0.016	0.2	0.31
ZS04	64	4	0.50	2	0.016	0.1	0.16

FLZ = fluconazole; MCZ = miconazole; ITZ = itraconazole; CLZ = clotrimazole; KTZ = ketoconazole; AMB = amphotericin B; 5FC = flucytosine

Amino acid variations of the P-450LDM from FLZ-resistant Candida albicans. The P-450LDM of *C. albicans* B311 consists of 528 amino acid residues with a calculated molecular mass of 60716 daltons. The highly conserved region at the amino terminal portion of the protein is the putative membrane anchoring region (23ISILLGVPFVYNLVWQYLYSL43) characterized by the presence of mainly hydrophobic amino acids. The highly conserved region at the carboxyl terminal portion of the protein (463FGGGRHRCIGEQFAYVQLGTI483) is the putative heme binding region 2, which is highly conserved in all known P-450LDMs. A comparison of the B311 P-450LDM sequence with that of the previously published sequence [10] revealed that it is 98.2% identical. The three changes between B311 and the previously published sequence were E116D, T128K and R287K of which two, E116D and R27K, are considered to be conserved substitutions. Assuming that the clinical isolate of *C. albicans* investigated by Lai and Kirsch and used for the sequencing of P-450LDM is susceptible to FLZ, the above three amino acid changes collectively do not contribute to the development of resistance to azoles, and in particular to FLZ [10]. Moreover, if we disregard the E116D, T128K and R287K alterations from the FLZ-resistant clinical isolates, there were seven amino acid changes detected in the LDM sequences of the six clinical isolates we evaluated, when compared to the sequence of B311.

The amino acid sequences of the P-450LDMs from clinical isolates were compared with that of the susceptible strain B311. The P-450LDMs from the FLZ-resistant clinical isolates in comparison to that from the susceptible strain showed two to seven amino acid changes located throughout the protein encompassing 10 different loci (Table 2). Approximately one-half of the changes are conserved substitutions (E116D, K143R, E266D, D278E, R287K), whereas five of the loci (T128K, R267H, S405F, G450E, G464S) contained non-conservative substitutions. R287K is the only amino acid substitution found in all six clinical isolates. Three of the five non-conserved substitutions (S405F, G450E, G464S) were located at or near the highly conserved putative heme binding region of the protein. The remaining non-conserved changes were found in the middle (R267H) and the amino terminal (T128K) regions of the protein. Although all six clinical isolates showed increased MICs for FLZ, along with varying degrees of cross-resistance to other azoles, no *in vitro* susceptibility pattern was strongly associated with a specific amino acid changes. However, the greater the number of amino acid substitutions detected, the greater the extent of azole cross-resistance. For example, isolates DC and F01A had the highest number of amino acid changes associated with increased degrees of cross-resistance to azoles. One or more of these changes individually or in combination with other amino acid changes, may affect the property of the enzyme such that it is refractory to the inhibitory effect of FLZ.

Table 2. Amino acid variations in the P450LDMs from fluconazole-resistant clinical isolates of *C. albicans.*

C. albicans isolates	Amino acid positions									
	116	128	143	266	267	278	287	405	450	464
B311	Glu	Thr	Lys	Glu	Arg	Asp	Arg	Ser	Gly	Gly
(Lai)	Asp	Lys					Lys			
DC	Asp	Lys	Arg	Asp	His	Glu	Lys			
F01A		Lys	Arg	Asp	His	Glu	Lys			
PL01A							Lys		Glu	Ser
RC			Arg				Lys			
ZW02		Lys					Lys	Phe		
ZS04			Arg				Lys			

Prediction of secondary structures of P-450LDMs from C. albicans. The predicted secondary structure as assessed by the ability to form α-helix conformations based on the model of Boscott and Grant [2] of P-450LDM from *C. albicans* B311 is shown in Figure 1. Four of the seven amino acid changes found in various FLZ-resistant clinical isolates, but not in the susceptible clinical and laboratory isolates, are located on the predicted α-helical regions of P-450LDM. Although three of the four changes (K143R, E266D and D278E) located on the α-helical regions are conserved substitutions, individually or collectively these alterations could contribute to the development of resistance to FLZ. At the present time, it is not clear if the observed in-vitro azole resistance of the clinical isolates is due to an alteration of the secondary structure of the protein initiated by one or more amino acid changes, or due to the alteration of amino acid residues that directly participate in the binding of the azole to the enzyme. The fact that a biologically functional P-450LDM was present in the FLZ-resistant isolates, suggest that the altered primary structure of the protein affected the susceptibility of the enzyme to FLZ without affecting its biological function.

Figure 1. Predicted secondary structure of P450LDM from *C. albicans* B311. The predicted α-helical regions of P450LDM as described by Boscott and Grant (1994) are shown in bold and underlined with the helix labels given at the end of each row. The seven amino acid variations found in the fluconazole-resistant *C. albicans* isolates are shown above the respective residue.

Itraconazole uptake studies. One of the common mechanisms of drug resistance frequently found in both pro- and eukaryotes is a decrease in the intracellular accumulation of the drug. There are two known mechanisms that limit the intracellular accumulation of unwanted molecules. One mechanism restricts the drug's entry into the cell by altering the permeability; the second is by extruding the accumulated drug by an efflux pump at the expense of energy, against a concentration gradient. The net result of either process is the reduction in the amount of intracellular drug in the cell and thus making the organism less susceptible to the drug. Resistance to FLZ in *C. albicans*, *Candida glabrata* and *Candida krusei* has been associated with reduced accumulation of FLZ, either due to reduced entry into the cell [8, 9] or due to extrusion by an efflux pump [20-22]. We therefore investigated the intracellular accumulation of ^{3}H-itraconazole in these azole-resistant clinical isolates, and compared the results with those obtained for the FLZ-susceptible *C. albicans* B311 strain. In both FLZ-susceptible and -resistant *C. albicans* isolates maximum accumulation of ^{3}H-itraconazole occurred within 60 min (Figure 2). A comparison of the amounts of ^{3}H-itraconazole accumulated in the six clinical isolates and the B311 showed no significant diffe-

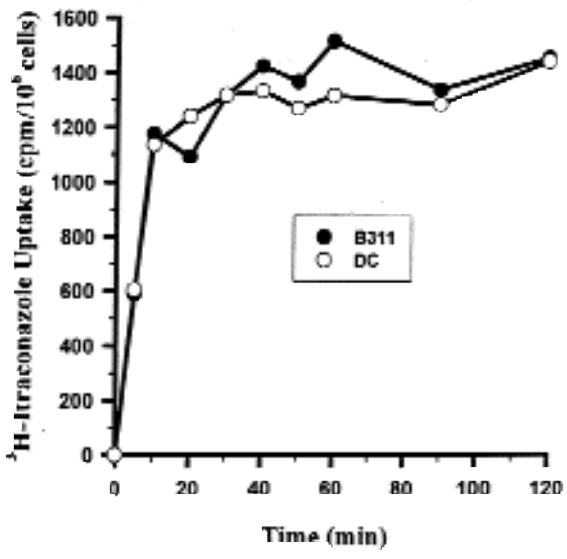

Figure 2. Uptake of itraconazole in the fluconazole-resistant (DC) and the fluconazole-susceptible (B311) *C. albicans.* The accumulation of itraconazole is expressed as cpm per 1 x 10^{6} cells.

rence (Table 3), thus suggesting that reduced accumulation may not be the primary reason for the reduced susceptibility of these *C. albicans* isolates to FLZ.

Table 3. Accumulation of ^{3}H-itraconazole in fluconazole-susceptible and -resistant isolate of *C. albicans.*

C. albicans isolates	^{3}H-Itraconazole uptake (cpm/1 x 10^{6} cells/h)
B311	1350 ± 383
DC	1256 ± 249
F01A	1578 ± 403
PL01A	1435 ± 315
RC	1653 ± 458
ZW02	1342 ± 284
ZS04	1397 ± 372

Estimation of P-450LDM gene dosage. It is possible that the observed resistance to FLZ is due to increased synthesis of P-450LDM either by the presence of multiple copies of the gene or by up-regulation of the synthesis of P-450LDM. Genome blot analysis revealed that both the susceptible and the resistant *C. albicans* have the same P-450LDM gene dosage (Figure 3). This observation precludes the possibility that FLZ resistance in the *C. albicans* isolates we studied is due to gene amplification. The possible up-regulation of P-450LDM synthesis in the resistant isolates was investigated by measuring the amount of P-450LDM mRNA in the resistant isolates by Northern blot analysis and compared it to that of the susceptible *C. albicans* B311. No significant difference in the amounts of mRNA specific to the P-450LDM was obtained between the resistant and the susceptible strains (data not shown). These results suggest that the possible mechanism of FLZ resistance in these clinical isolates of *C. albicans* is not the increased synthesis of P-450LDM, and thus, may be mediated by an altered P-450LDM refractory to the inhibitory action of FLZ.

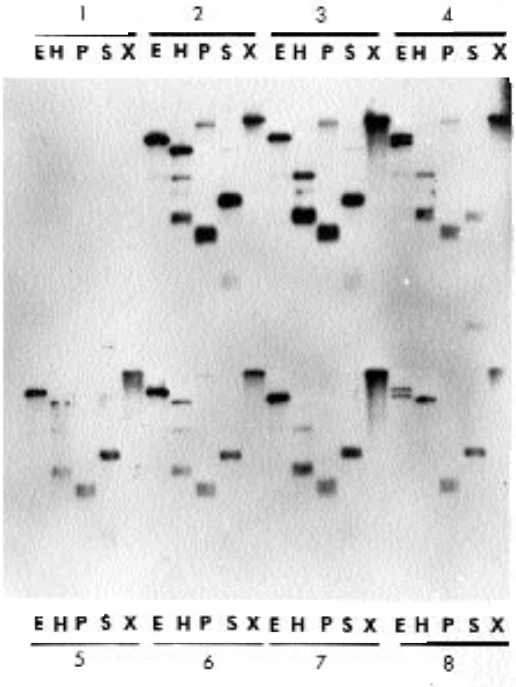

Figure 3. Genome blot analysis for the estimation of P450LDM gene dosage. Legends: Panel 1, *Saccharomyces cerevisiae*; Panel 2, *C. albicans* B311; Panels 3-8, Fluconazole-resistant *C. albicans* DC, F01A, PL01A, RC, ZW02 and ZS04, respectively. E = *Eco*R1; H = *Hind*III; P = *Pst*1; S = *Sty*I; X = *Xho*I.

DISCUSSION

In vitro resistance to FLZ among clinical isolates of *C. albicans* isolated from AIDS patients suffering from esophageal candidiasis is a more common occurrence in recent years [7,17]. Failure of FLZ therapy against mucosal candidiasis due to *C. albicans* in AIDS patients is estimated to be as high as 30% [1,4,18]. Thus, an understanding of the mechanism(s) of resistance to FLZ in *C. albicans* is not only important in the design and development of new antifungals, but is also important in the selection of the appropriate antifungal at the earliest possible time. We therefore investigated several possible mechanisms for the reduced susceptibility to FLZ among the six clinical isolates of *C. albicans* obtained from AIDS patients in the Detroit Medical Center, Detroit, Michigan. These results ruled out the possibility that the resistance in these isolates is due to reduced accumulation of the drug in the cell or due to increased expression of P-450LDM. On the other hand, the several amino acid variations found in the P-450LDM from the resistant clinical isolates suggest that the mutant enzyme in these isolates may play role in conferring resistance to FLZ.

Since the pre-treatment clinical isolates in our study were not available, we compared the P-450LDM sequence of the resistant isolates with that of the FLZ-susceptible laboratory strain B311. A comparison of the sequence of B311 to the previously published P-450LDM sequence [10] from a clinical isolate showed 10 nucleotide changes. Three of these changes resulted in amino acid alterations (E116D, T128K, R287K). Since both B311 and the clinical isolate of *C. albicans* used by Lai and Kirsch are susceptible to FLZ these amino acid variations by themselves are not contributory to the FLZ resistance. However, it is possible that these amino acid changes in combination with other amino acid alteration could be responsible for the FLZ-resistance. If amino acid variations common to both B311 and the published sequence are eliminated, only seven amino acid changes (K143R, E266D, R267H, D278E, S205F, G450E and G464S) were found in the six isolates and they belonged to four groups. In addition, it appears that the published P-450LDM sequence is more closely related to those of our clinical isolates than to B311.

Since radio-labelled FLZ was not available for experimentation, we substituted ^{3}H-itraconazole to study the accumulation of azole in the resistant clinical isolates. The pleiotropic drug resistance (PDR) determinant responsible for the active expulsion of FLZ from the cell is capable of effluxing a variety of structurally unrelated compounds [20-22]. Therefore, we expected that if the resistance in the clinical isolates was mediated through efflux, itraconazole accumulated by the cell should be pumped out by the efflux protein and make the cells less susceptible to the drug. In addition, we also examined the accumulation of rhodamine 6G in the resistant cells and compared the results with those obtained for B311 using a procedure described previously [23]. No significant difference was found in rhodamine accumulation between the FLZ-resistant clinical isolates and the susceptible B311. These data also suggest that the mechanism of resistance to FLZ in these isolates is not due to reduced accumulation of the drug.

Our characterization of the P-450LDM from the six different FLZ-resistant clinical isolates showed multiple amino acid changes in all the isolates when compared to the susceptible strain. It is not clear at present whether multiple mutations are required for the emergence of resistance. A more refined structure-function analysis of

P-450LDM by site-directed mutagenesis is required to delineate the role of each variant amino acid with respect to the resistance to FLZ. However, analysis of the amino acid sequence of P-450LDM isolated from *C. albicans* isolates resistant to FLZ from other geographical regions [11] have demonstrated three of the same mutations (E266D, G450E, and G464S) as we found, thus suggesting that these mutations evolved independently and perhaps play a significant role in FLZ resistance. More recently, Sanglard and collaborators [24] have also described several amino acid substitutions in the CYP51A1 recovered from *C. albicans* clinical isolates with documented azole resistance. In fact, the investigators were able to describe several amino acid mutations (Y132H, S405F, G464S, and R467K) which had measurable effects on the affinity of the target enzyme for the azoles evaluated. Of these four amino acid substitutions, two of the mutations (S405F and G464S) are the same mutations we were able to identify in the clinical isolates we evaluated. Additionally, Sanglard and collaborators also demonstrated that the mutation S405F, which was also one of the mutations we were able to document, was found as a single amino acid substitution in a CYP51A1 gene recovered from an azole resistant *C. albicans*.

Alteration(s) of the primary structure of a protein at times results in the alteration of the secondary structure of the protein. In such cases, the observed phenotypic variation(s) is due to the alteration of the secondary structure of the protein and not directly associated with specific amino acid change. The predicted secondary structure of the P-450LDM from the resistant isolates failed to show any detectable change, suggesting that altered amino acid may play a direct role in the resistance trait. Since azole antifungal agents bind to the active site of P-450LDM [30] by mimicking the natural substrate, it is possible that these amino acid changes may interfere with the initial binding of azoles to the active site of the enzyme thus making it highly refractory to the inhibitory action of FLZ.

The authors wish to thank the National Science Foundation (Grant HRD 92-53037) for their support. We also wish to thank Janssen Pharmaceutica for their gracious donation of the radiolabelled itraconazole.

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