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Hypothesis on the Mechanism of Resistance to Fluconazole in *Histoplasma capsulatum*

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An AIDS patient with disseminated histoplasmosis who improved during treatment with fluconazole but remained fungemic and subsequently relapsed is described. Isolates obtained from blood during therapy showed a progressive increase in fluconazole MIC from 0.625 to 20 μg/ml. The pretreatment, or parent, isolate and the posttreatment, or relapse, isolate demonstrated identical genetic patterns by PCR fingerprinting with three different primers. Fluconazole was a less potent inhibitor of the growth of the relapse isolate than of the pretreatment isolate (50% inhibitory concentration [IC_{50}] = 11.7 μM versus 30.6 μM), while itraconazole was more potent (relapse isolate IC_{50} = 0.0011 μM versus pretreatment isolate IC_{50} = 0.0064 μM). Neither the increased sensitivity to itraconazole nor the decreased activity of fluconazole on the growth of the relapse isolate results from changes in the intracellular content of these agents. To reach 50% inhibition of ergosterol synthesis in both the parent and relapse isolates, about 2 nM itraconazole was needed; with fluconazole, 50% inhibition was achieved at 20.9 μM and 55.5 μM, respectively. Resistance to fluconazole may develop during treatment and results from decreased sensitivity of ergosterol synthesis.

Disseminated histoplasmosis is a progressive fatal infection in patients with AIDS. Although treatment is effective, recurrence is common, mandating chronic antifungal suppression to prevent relapse. Amphotericin B is the most effective therapy and the treatment of choice for more severe cases (12). Itraconazole was effective therapy for patients with mild or moderate clinical manifestations, inducing remission in 85% of cases (10). Itraconazole also was highly effective as maintenance therapy, preventing relapse in 95% of patients (10, 11). High-dose treatment with fluconazole (800 mg daily) induced remission in 74% of patients, but nearly one-third relapsed during maintenance treatment with 400 mg daily (13).

The poorer response to fluconazole may be caused by reduced in vitro activity for *H. capsulatum*. Treatment failure may have been caused by development of resistance during therapy in some patients. In this report we describe mechanisms for acquired resistance in isolates from one of these patients.

CASE REPORT

A 39-year-old human immunodeficiency virus-infected male presented with a 4-week history of fever, cough, dyspnea, and weight loss. Abnormal physical findings included a temperature of 37.8°C and mild tachypnea (respiratory rate, 24/min). Chest radiograph showed diffuse interstitial infiltrates. Laboratory abnormalities included a hemoglobin of 9.1 g/dl, pO^2 of 65 mm Hg, alkaline phosphatase of 309 IU/dl, aspartate aminotransferase of 128 IU/dl, and albumin of 3.1 g/dl. The presumptive diagnosis of histoplasmosis was made when organisms resembling *H. capsulatum* var. *capsulatum* were seen by fungal stain of bronchoalveolar lavage fluid and subsequently proven by isolation from the lavage fluid and blood.

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Treatment was started with fluconazole (600 mg daily) through an investigational protocol (AIDS Clinical Trials Group 174/Mycoses Study Group 23 protocol). The patient defervesced and gained 5 kg over the next 2 months. However, one colony of *H. capsulatum* var. *capsulatum* was isolated from protocol-mandated blood cultures taken at weeks 8 and 12 of therapy.

At week 16, fever to 39.4°C recurred, and examination revealed splenomegaly. Blood cultures now grew numerous colonies of *H. capsulatum* var. *capsulatum*. Fluconazole serum concentration was 37.4 μg/ml. Fluconazole was discontinued, and amphotericin B (50 mg) was started daily for two weeks and every other day for another 6 weeks, followed once weekly for 4 months. Maintenance treatment was changed to itraconazole (200 mg twice daily) for an additional 2 years without recurrence of histoplasmosis or isolation of *H. capsulatum* var. *capsulatum* from cultures.

MATERIALS AND METHODS

MIC determination. Susceptibility was measured by the standardized method for yeasts developed by the National Committee for Clinical Laboratory Standards (1) modified for *H. capsulatum* by extending the incubation to 96 to 144 h. Yeast cells were suspended in sterile normal saline and adjusted to a 5 McFarland standard spectrophotometrically at A_{530}. Yeast suspensions were diluted 1/100 in RPMI 1640 medium containing L-glutamine and buffered to pH 7.0 with 0.165 M [3-(*N*-morpholino)propane sulfonic acid] (MOPS) (Whittaker MA Bioproducts). Stock solutions of drugs were made at the following concentrations: amphotericin B, fluconazole, and ketoconazole, 16 mg/ml in dimethyl sulfoxide (DMSO); itraconazole, 5 mg/ml in polyethylene glycol (M_r = 200). Dilutions of these stocks in RPMI were dispensed in tubes (12 by 75 mm) and the test organisms were added. After 96 to 144 h, depending upon the turbidity in the drug-free control, MICs were determined visually. The MIC was defined as the concentration of drug at which at least 80% growth inhibition occurred.

Culture methods. *H. capsulatum* isolates were maintained on GY (glucose–yeast extract–L-cysteine–agar, 2%–1%–0.06%–2%) slants at 37°C for the experiments measuring protein and sterol content and sterol synthesis (7). A loopful from a week-old slant was inoculated in 100 ml of GY medium (glucose–yeast extract–L-cysteine, 2%–1%–0.03%) containing 20 U of penicillin and 40 μg of streptomycin per ml and cultivated in 500-ml Erlenmyer flasks at 37°C for 1 week in a reciprocal shaker. Aliquots (0.5 ml) of this culture were used to inoculate 100

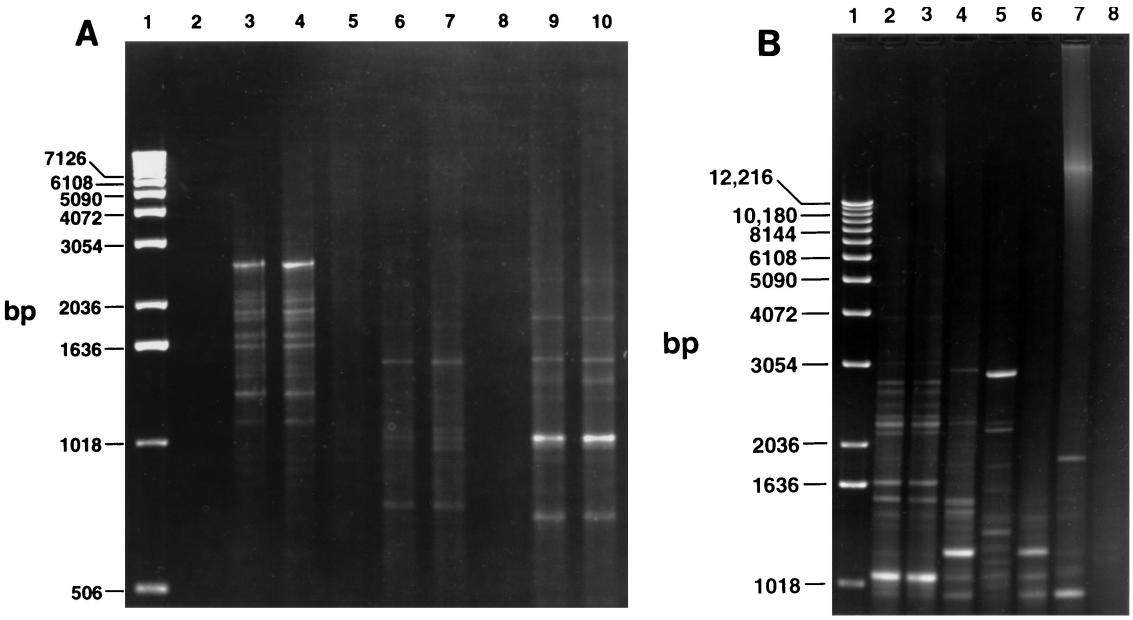

FIG. 1. (A) PCR of the patient's parent and relapse isolates with primer 1 (lanes 3 and 4), primer 2 (lanes 6 and 7), and primer 3 (lanes 9 and 10). (B) PCR amplification pattern for the patient's baseline isolate and standard control strains of *H. capsulatum* var. *capsulatum* with primer 3. Lanes: 1, molecular weight markers; 2 through 8, DNA from the patient's parent isolate (lane 2) and relapse isolate (lane 3) and from control isolates G217b (lane 4), G186 (lane 5), G184 (lane 6), G222 (lane 7), and Downs (lane 8); 9, H_2O blank.

ml of GY medium in 500-ml Erlenmeyer flasks. Cells were grown at 37°C aerobically in a reciprocating shaker for 72 h.

To evaluate the effects of itraconazole and fluconazole on growth, the azole derivatives and/or solvent (DMSO [final concentration, 0.1%]) were added to the GY medium prior to inoculation with 0.5-ml aliquots of the above-described culture (7). After 48 h of growth at 37°C in a reciprocating shaker, cells were collected by centrifugation and washed twice in physiological saline. The pellet was suspended in 3 ml of water and added to 2 g of glass beads (diameter, 0.45 mm). Cells were shaken vigorously for 20 cycles of 4 s with 26-s intermittent cooling. The homogenate was separated quantitatively from the glass beads and diluted to 15 ml (7). Protein content of the homogenates was determined by the Biorad method, with bovine albumin used as the standard.

Sterol synthesis. To study sterol biosynthesis in *H. capsulatum*, 100 ml of GY medium was supplemented with 5 μCi of [^{14}C]acetate (specific activity, 58 μCi/mmol) and different concentrations of azole antifungal and/or DMSO immediately before inoculation. Inoculation, growth conditions, cell harvest, and homogenization were as described above. The homogenate was supplemented with an equal volume of 15% KOH in 90% ethanol. The mixture was heated for 1 h at 85°C in a water bath and cooled, and the nonsaponifiable lipids were extracted with 1 volume of *n*-heptane (Spectrograde). Total radioactivity in the heptane extract was determined by liquid scintillation counting with Opti-phase (Pharmacia) as a scintillant (7). The ergosterol content of the heptane extracts of cell homogenates was determined by measuring the absorption difference between 292 and 282 nm. An ergosterol standard curve was used to calculate the ergosterol contents. To separate, quantify, and identify the sterols, the heptane extracts were dried under a stream of nitrogen and dissolved in minimal volumes of methanol-water (95:5). Sterols were separated by high-performance liquid chromatography (HPLC) on a Varian 9010 liquid chromatograph equipped with a Varian 9095 automatic injector, a Varian 9065 Polychrom Detector, and a Berthold LB507 HPLC radioactivity monitor with Pico Aqua (Canberra Packard) as a scintillant and connected with a Compaq 386/33 computer (8). Sterols were identified according to their retention times relative to those of standards and/or by gas chromatographic-mass spectrometric analysis (8).

Intracellular content. Cells were grown for 24 h (exponential phase) in 500-ml Erlenmeyer flasks, containing 100 ml of GY medium, in a reciprocating shaker at 37°C. To these cultures, 300 nM [^{3}H]itraconazole (specific activity, 15.0 mCi/mmol; Janssen Research Foundation) or 300 nM [^{14}C]fluconazole (specific activity, 4.81 mCi/mmol; gift from Pfizer Central Research, Sandwich, United Kingdom) were added. After 1 h of incubation at 37°C, cells were collected by centrifugation and washed three times with ice-cold saline containing 10 μM unlabelled azole. Cells were homogenized as described above, and the homogenates were digested with hydrogen peroxide-perchloric acid (4), and the radioactivity was determined as described previously (5).

PCR. The isolates were grown in the mould phase in potato dextrose broth (Difco Laboratories, Detroit, Mich.). The mould cultures were ground under liquid nitrogen to degrade the cellular structure and release the nucleic acids. The isolates were further lysed in 50 mM Tris–62.5 mM EDTA–2% sodium dodecyl sulfate. The lysates were then purified through cesium chloride gradients and phenol-chloroform-isoamyl alcohol extractions. The purified DNA was checked for concentration by agarose gel electrophoresis prior to amplification. Three distinct primers previously shown to have high discriminatory power for *Histoplasma* (≥27 nt) were used (3). Sequences for the primers were as follows: H1, GGCCATAGAGTCTTGCAGACAAACTGC; H2, AACGTTCATGATA ACTTCTGCTCTTCATCG; H3, AAGCTTGCATTTGTGTTCCTTGATAAG TG. The amplification was performed in a 9600 thermocycler (Perkin Elmer) by using standard PCR mixes. Agarose gel electrophoresis was performed in a 1.3% gel at 70 V for 4 h and visualized with 8 μl of ethidium bromide at 10 mg/ml. The DNA bands were visualized and photographed under UV light.

RESULTS

MICs. Numerous colonies were present at base line, but only a single colony was noted at weeks 8 and 12 of therapy, when the patient appeared to be improving clinically. Numerous colonies again were isolated at relapse at week 16, suggesting that the isolate had become resistant to fluconazole. Fluconazole MICs were 0.62 μg/ml for the pretreatment isolate (parent isolate), 1.25 μg/ml at week 8, 2.5 μg/ml at week 12, and 20 μg/ml at week 16 (relapse isolate). Itraconazole MICs were 0.004 μg/ml for all isolates. The fluconazole blood concentration on a specimen taken during the last week of therapy was 37.4 μg/ml.

PCR fingerprinting. PCR fingerprinting with three random primers showed identical amplification patterns for the pretreatment and the relapse isolate (Fig. 1a). The patterns were different from those obtained with several unrelated *H. capsulatum* strains. Results using primer 3 are shown in Fig. 1b. This indicates that the parent and the relapse isolate are clonal.

Effects of itraconazole and fluconazole on growth and ergosterol content. Growth was measured by determining the protein content of cells collected after 48 h of growth in the presence of fluconazole or itraconazole. Compared with its effect on growth of the pretreatment (parent) isolate (50% inhibitory concentration [IC_{50}] = 11.7 μM [or 38.4 μg/ml based on a molecular weight of 306; 1 μM = 0.3 μg/ml]),

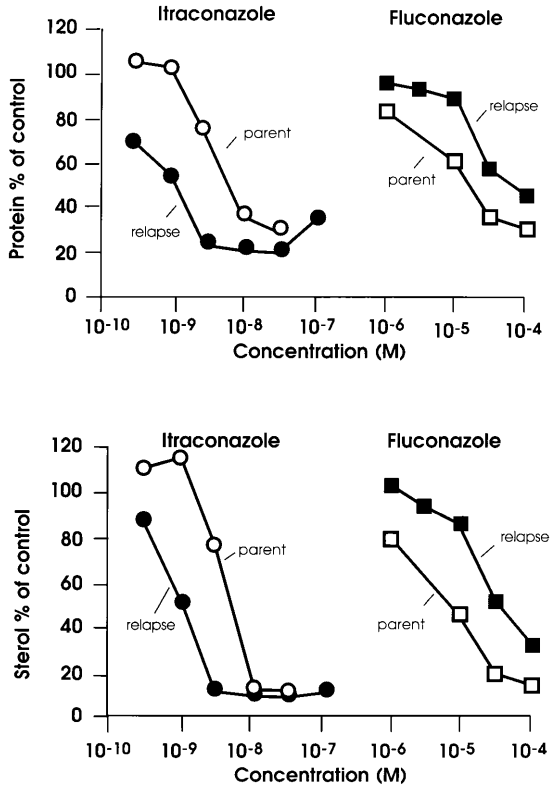

FIG. 2. Effects of fluconazole and itraconazole on the protein and ergosterol content of parent and relapse isolates. Growth of *H. capsulatum* was measured by determining the protein content of cells collected after 48 h of growth in the presence of fluconazole or itraconazole. Ergosterol content was measured by UV spectroscopy of parent and relapse isolates grown for 48 h.

fluconazole was a three-times less-potent inhibitor of the growth of the relapse isolate (IC_{50} = 30.6 μM [100 μg/ml]) (Fig. 2). However, itraconazole was a six-times more-potent growth inhibitor of the relapse isolate (IC_{50} = 0.0011 μM [0.0016 μg/ml based on a molecular weight of 707; 1 μM = 0.7 μg/ml]) than of the pretreatment isolate (IC_{50} = 0.0064 μM [0.009 μg/ml]).

Similar results were found when ergosterol content was measured spectrophotometrically (Fig. 2). A 50% decrease in ergosterol content of the relapse isolate required 0.001 μM itraconazole (0.0014 μg/ml), whereas 0.0048 μM (0.0068 μg/ml) was needed to reach a 50% decrease of the ergosterol content of the pretreatment isolate. With fluconazole the IC_{50} was five times higher for the relapse isolate (IC_{50} = 25.8 μM [84.3 μg/ml]) than for the pretreatment isolate (IC_{50} = 5.3 μM [17.3 μg/ml]).

Effects on ergosterol biosynthesis. Ergosterol and ergosta-5, 22-diene-3-β-ol are the main sterols formed from [^{14}C]acetate in both parent and relapse isolates (Fig. 3). In the pretreatment isolate, itraconazole was a more potent inhibitor of ergosterol synthesis than fluconazole (IC_{50} = 0.0017 μM [0.002 μg/ml] versus 20.9 μM [68.3 μg/ml], respectively). In the relapse strain, whereas 0.0018 μM itraconazole (0.0025 μg/ml) continued to inhibit ergosterol synthesis by 50%, the IC_{50} of fluconazole rose to 55.4 μM (181.04 μg/ml). In the relapse isolate, substantial synthesis of both ergosterol (114,000 dpm/100-ml cell suspension) and ergosta-5,22-diene-3-β-ol (67,000 dpm/100-ml cell suspension) took place even at a 100 μM fluconazole concentration (326.80 μg/ml) (Fig. 3). Ergosterol synthesis was almost completely blocked at that concentration in the pretreatment isolate.

Concomitant with the inhibition of ergosterol and ergosta-5, 22-diene-3-β-ol synthesis, obtusifolione [4,14-dimethyl-ergosta-8,24(28)-diene-3-one] and eburicol (24-methylene-dihydrolanosterol) accumulated (Fig. 3). This suggests that these azole antifungals inhibited in *H. capsulatum* not only the 14α-demethylase (eburicol was the substrate of this P-450-dependent enzyme) but also the 3-ketosteroid reductase (an enzyme of the 4-demethylation pathway; Fig. 4). At 1 nM itraconazole almost no obtusifolione or eburicol was found in the pretreatment isolate, whereas in the relapse isolate significant amounts of obtusifolione accumulated (Fig. 3). This suggests that the 3-ketosteroid reductase and the 14α-demethylase became more sensitive to itraconazole. However, compared with the enzymes in the pretreatment isolate, those in the relapse isolate became less sensitive to fluconazole.

Intracellular content. After 1 h of incubation of exponentially growing yeast cells in the presence of [^{3}H]itraconazole or [^{14}C]fluconazole, no substantial differences were found in the intracellular contents between the pretreatment and the relapse isolates (Table 1). For itraconazole, an intracellular content of 0.262 nmol/mg of protein was found in the pretreatment strain compared to 0.298 nmol/mg of protein in the relapse strain. The concentrations for fluconazole were 0.082 and 0.103 nmol for pretreatment and relapse isolates, respectively.

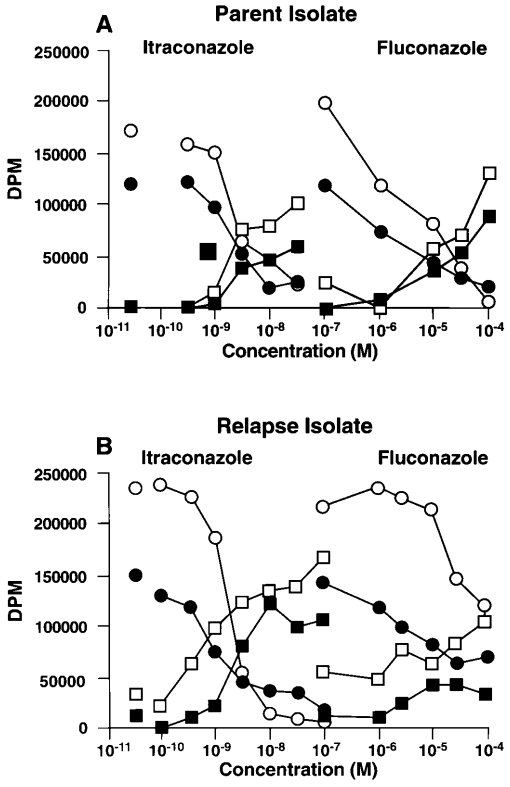

FIG. 3. Effects of itraconazole (ITZ) and fluconazole (FCZ) on ergosterol synthesis from [^{14}C] acetate by the patent and relapse isolate. Time of incubation was 48 h in GY medium. Drug and [^{14}C] acetate were added immediately before inoculation. Sterols formed are: ergosterol (○), ergosta-5, 22-diene-3-ol (●), obtusifolione (□), and eburicol (24-methylene-dihydrolanosterol) (■). Results for controls (ergosterol synthesis in the presence of solvent DMSO) are depicted inside the left-hand margin by the datum points that are not connected by lines. Results are mean values from four experiments.

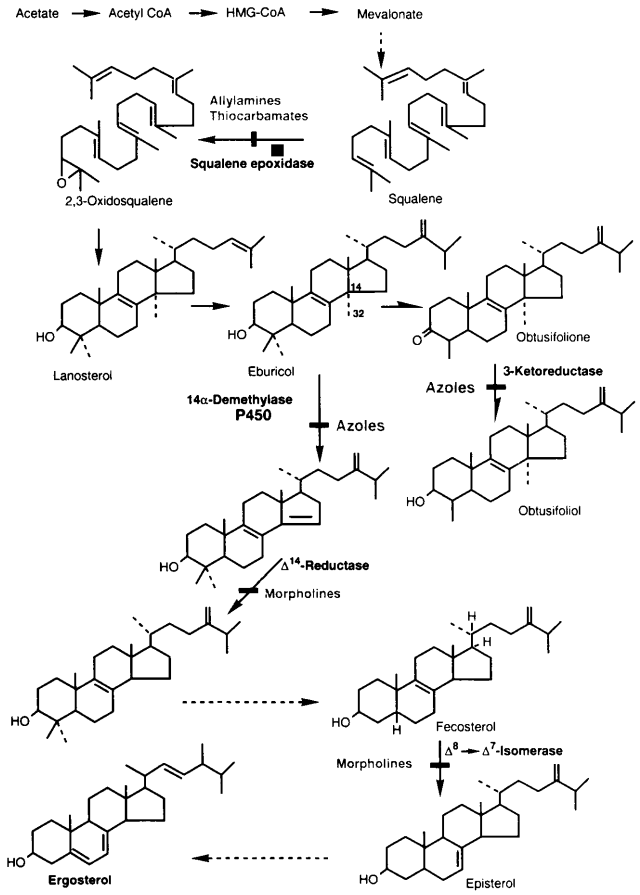

FIG. 4. Ergosterol biosynthesis pathway. Azole antifungals inhibit the cytochrome-P-450 dependent 14α-demethylase. This coincides with the accumulation of lanosterol, eburicol, and obtusifoliol. In *H. capsulatum* the 3-ketosteroid obtusifolione also accumulates. From Vanden Bossche et al., *Trends in Microbiology*, 1994, with permission of the publisher (9).

DISCUSSION

Fluconazole is a less effective treatment for histoplasmosis than is amphotericin B or itraconazole. Relapse is more common with fluconazole than with amphotericin B or itraconazole (11a). A likely reason for fluconazole's inferior efficacy in histoplasmosis appears to have been its reduced antifungal activity for *H. capsulatum* var. *capsulatum*. Typical MICs are 50-fold higher with fluconazole (0.62 to 1.25 μg/ml) than itraconazole (≤0.019 μg/ml) (14). Also, resistance may arise during fluconazole therapy.

One possible cause for the higher MICs of pretreatment isolates of *H. capsulatum* var. *capsulatum* to fluconazole than itraconazole is reduced uptake or more rapid efflux of fluconazole from the fungal cell. The intracellular content of fluconazole was threefold lower than that of itraconazole in the pretreatment and relapse isolates (Table 1). However, a change in intracellular content of fluconazole or itraconazole was not observed in the relapse isolate as compared to the pretreatment isolate. Thus, neither the increased susceptibility to itraconazole nor the decreased susceptibility to fluconazole in the relapse isolate results from changes in intracellular content of these triazole antifungal agents.

Reduced susceptibility of ergosterol synthesis to fluconazole appears to explain the inducible resistance noted in the relapse isolate (Table 1). Reduced accumulation of eburicol and obtusifolione suggests that the cytochrome P-450-dependent 14α-demethylase and 3-ketosteroid reductase became less sensitive to fluconazole.

Cross resistance between fluconazole and itraconazole was not observed. Of 16 patients who failed treatment with fluconazole in a clinical trial, MICs to fluconazole increased at least fourfold in 9 (56.2%) failure isolates. Itraconazole MICs remained ≤0.019 μg/ml in all but one of these failure isolates and was 0.077 μg/ml in that isolate. MICs above 0.077 μg/ml to itraconazole were not observed in 67 primary isolates and 16 failure isolates tested, including 10 failure isolates for which MICs to fluconazole were 10 μg/ml or more. This disparity highlights the difference in modes of action of itraconazole and fluconazole.

Interestingly, while the relapse isolate showed reduced antifungal activity with fluconazole, the activity of itraconazole was even greater (Table 1). The IC_{50} for protein and ergosterol content was significantly lower for the relapse than the pretreatment isolate, as was that for ergosterol synthesis. Induction of large amounts of obtusifolione by low concentrations of itraconazole in the relapse isolate suggests that the 3-ketosteroid reductase of the relapse isolate is more susceptible to itraconazole than is that of the parent isolate. Since 3-ketosteroids, such as obtusifolione, may destabilize the lipid bilayer and increase the fragility of membranes (2, 6), the increased amounts of obtusifolione formed in the relapse isolate incubated in the presence of itraconazole may explain the enhanced sensitivity to this triazole derivative.

In conclusion, this case illustrates the risk for relapse of histoplasmosis during treatment with fluconazole. Acquisition of resistance to fluconazole caused by reduced sensitivity of ergosterol synthesis appears to be responsible for the increased MIC of the relapse isolate. Cross resistance did not occur with itraconazole. Additional studies are needed to elucidate the mechanism of fluconazole resistance. Investigation of the effects on P-450-dependent 14α-demethylase and 3-ketosteroid reductase will establish the role of these *Histoplasma* enzymes in the mechanism of action and development of resistance to these azole derivatives.

TABLE 1. Effect of itraconacole and fluconazole on the parent and relapse isolates

Isolate	Itraconazole					Fluconazole				
	MIC (μg/ml)	Content (IC_{50} [μM])		Ergosterol synthesis (IC_{50} [μM])[a]	Intracellular triazole content (nmol/mg of protein)	MIC (μg/ml)	Content (IC_{50} [μM])		Ergosterol synthesis (IC_{50} [μM])[b]	Intracellular triazole content (nmol/mg of protein)
		Protein[a]	Ergosterol[a]				Protein[b]	Ergosterol[b]		
Parent	0.004	0.064	0.0048	0.0017	0.262	0.625	11.7	5.3	20.9	0.082
Relapse	0.004	0.0011	0.001	0.0018	0.298	≥20.00	30.6	25.8	55.4	0.103

[a] Itraconazole: 1 μM = 0.7 μg/ml; molecular weight = 705.
[b] Fluconazole: 1 μM = 0.3 μg/ml; molecular weight = 306.

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