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Fluconazole Resistance in *Candida glabrata*

C. A. HITCHCOCK,[1]* G. W. PYE,[1] P. F. TROKE,[1] E. M. JOHNSON,[2] AND D. W. WARNOCK[2]

Department of Discovery Biology, Pfizer Central Research, Sandwich, Kent CT13 9NJ,[1] and Department of Microbiology, Bristol Royal Infirmary, Bristol BS2 8HW,[2] United Kingdom

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We report a case of infection with *Candida glabrata* in which the organism became resistant to fluconazole and in which pre- and posttreatment isolates were available for comparison. The organism was cross-resistant to ketoconazole and itraconazole, in common with other azole-resistant yeasts. Fluconazole was a potent inhibitor of cytochrome P-450-dependent 14α-sterol demethylase ($P\text{-}450_{DM}$) in lysates of cells from both susceptible and resistant cultures (50% inhibitory concentration, 0.2 μM), indicating that resistance was unrelated to changes in $P\text{-}450_{DM}$. Instead, it appeared to arise from a permeability barrier to fluconazole, since resistant cells were unable to take up radiolabelled drug.

The azole (N-substituted imidazole or triazole) class of antifungal antibiotics is used commonly in the treatment of both superficial and deep-seated mycoses, including candidiases (6). These drugs most probably work by inhibiting ergosterol biosynthesis in fungal cells by binding to P-450-dependent 14α-sterol demethylase ($P\text{-}450_{DM}$), an important enzyme in ergosterol biosynthesis (7). This leads to the accumulation of methylated sterols, which are thought to disrupt membrane structure and function (27).

Clinical isolates of *Candida* vary widely in their susceptibilities to azoles, but only a small number of resistant strains have been isolated from treatment failures during more than 20 years of widespread use. In 1978, Holt and Azmi (14) described a case of candidiasis in a neonate in which resistance to miconazole developed in a strain of *C. albicans* following 9 weeks of treatment for a urinary tract infection. Since then, four cases have been reported. In those cases prolonged ketoconazole treatment for chronic mucocutaneous candidiasis led to resistance in the infecting *C. albicans* strains (15, 20, 25). Reports of fluconazole resistance are of very low incidence because the drug has been used to treat more than 15 million patients, including more than 250,000 patients infected with the AIDS virus. The small number of cases of resistance have been described following the treatment of candidiases in a seriously debilitated patient with hepatorenal failure (24) and in immunocompromised patients (4), including those with AIDS (5, 16, 19, 26).

The known mechanisms of azole resistance in *C. albicans* may be divided broadly into two types: resistance caused by the decreased azole susceptibility of $P\text{-}450_{DM}$, the target enzyme in ergosterol biosynthesis (9, 20), or permeability resistance in cells unable to take up drug (8, 13, 18). A recent report described both mechanisms in a fluconazole-resistant clinical isolate of *C. glabrata* (23). Here we report a patient infected with *C. glabrata*. The organism appeared to become resistant after 2 weeks of oral treatment with fluconazole, and pre- and posttreatment isolates were available for comparison. Like most azole-resistant *C. albicans* and *C. glabrata* strains, the isolate described here was cross-resistant to other azoles, including ketoconazole and itraconazole, and the mechanism of resistance appeared to be reduced drug uptake rather than changes at the level of ergosterol biosynthesis.

* Corresponding author.

CASE REPORT

A 69-year-old woman was admitted to the hospital on 10 October 1989 with bilateral pneumonia, anemia, and mild dysuria. She deteriorated further, and on 11 October she was transferred to the intensive therapy unit for ventilation, where she then suffered a large hematemesis. Laparotomy and gastrotomy revealed two chronic, benign gastric ulcers which were repaired. During the following week she remained ventilator dependent and was noted to have bilateral pleural effusions sufficient to warrant chest drain insertion on 17 October. This resulted in hemorrhage which necessitated thoracotomy to repair the damaged right lung; several empyemas were drained. She was again ventilated and, as a further complication, a computed tomography scan of her abdomen on 25 October showed a collection of fluid. Laparotomy revealed a splenic tear, and splenectomy was performed. A tracheostomy was also performed on 26 October.

Four urine specimens taken between 16 and 23 October yielded *C. glabrata* (Fig. 1). Intermittent local instillation of amphotericin B was commenced on 24 October. Blood cultures taken from an arterial line on 25 and 29 October yielded *Rhodotorula glutinis*, and because the patient was febrile and colonized with *C. glabrata*, parenteral treatment with fluconazole (200 mg daily) was commenced on 30 October; local treatment with amphotericin B was discontinued. Four urine specimens taken during the following week were sterile, but *R. glutinis* was isolated from a blood culture taken on 3 November. However, subsequent blood cultures were sterile. Fluconazole treatment was discontinued on 12 November. In the meantime, the patient's condition had improved, enabling artificial ventilation and parenteral nutrition to be discontinued. However, urine and sputum specimens and throat and rectal swabs taken on 13 and 16 November revealed that she was still colonized with *C. glabrata* (Fig. 1). No further antifungal treatment was given, and the patient was discharged to a geriatric hospital on 5 December.

MATERIALS AND METHODS

Materials. Solvents and other chemicals were of analytical grade and were purchased from BDH or Sigma. The following radiochemicals were synthesized by Amersham International: DL-[2-^{14}C]mevalonic acid (dibenzyldiethyldiamine salt; specific radioactivity, 1.89 GBq/mmol), [2-^{14}C]acetic

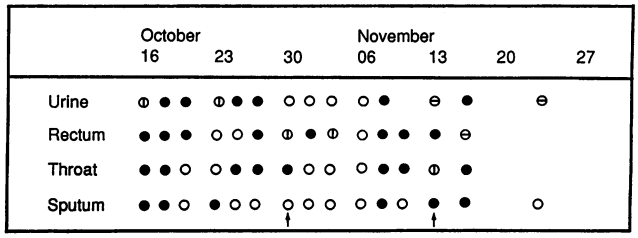

FIG. 1. History of isolation of *C. glabrata* from various clinical sites of the case patient between 16 October and 27 November 1989. ●, *C. glabrata* isolated; ↑, fluconazole therapy; Ⓓ, *C. glabrata* isolated (MIC, 12.5 μg/ml); ⊖, *C. glabrata* isolated (MIC, 100 μg/ml); ○, nothing isolated.

acid (specific radioactivity, 1.85 GBq/mmol), and [^{3}H] fluconazole (specific radioactivity, 81.4 GBq/mmol).

Isolation and identification of *C. glabrata* from the patient. Samples from the patient were cultured on a range of media incubated at 37°C. Isolates of *C. glabrata* were identified by means of ID 32C identification strips (BioMerieux).

Cultures of *C. glabrata* isolated from urine (fluconazole susceptible, Y33.90; fluconazole resistant, Y33.91) were maintained in freeze-dried ampoules and were subcultured on slopes of Sabouraud dextrose agar before use. Late-exponential-phase cultures were grown in High Resolution Medium (HR; Oxoid) essentially as described previously (8). The organisms were typed by restriction fragment length polymorphism (RFLP) analyses of genomic DNA (21). The resulting RFLP patterns were qualitatively different, thereby suggesting that the organisms were clonally unrelated.

Azole susceptibility. The MICs of fluconazole, ketoconazole, and itraconazole for the *C. glabrata* isolates were measured by broth macrodilution in HR medium (17). The MIC was defined as the lowest drug concentration at which there was no visible growth.

Measurement of sterol biosynthesis in cells and lysates. Sterol biosynthesis was assayed by the incorporation of [^{14}C]mevalonic and [^{14}C]acetic acids into the nonsaponifiable lipid fraction (NSF) of lysates and cells, respectively (1, 12).

Measurement of fluconazole uptake. The ability of susceptible and resistant cells to take up [^{3}H]fluconazole was measured by a filter-based assay (10). Cells (2×10^8) were incubated with 0.1 nmol of radiolabel in 1 ml of HR medium. Control experiments with autoclave-killed cells and blank assays without cells were done in order to establish the amount of drug binding to cells. These amounts were reproducible for both strains and did not exceed 10% of the incorporated radioactivity.

RESULTS

Effect of fluconazole on cell growth. When tested by HR broth dilution assays, the resistant culture showed greatly reduced susceptibilities to fluconazole, ketoconazole, and itraconazole in comparison with those of the susceptible culture (Table 1).

Effects of fluconazole on sterol 14α-demethylation. When cell lysates were incubated with [^{14}C]mevalonic acid, approximately 40% of the total radioactivity in the assay was recovered in the NSF for both susceptible and resistant organisms. Sterols were separated from the other NSF components and were fractionated into desmethylated and 14α-methylated classes by one-dimensional thin-layer chromatography by previously published methods (12). The desmethylated and 14α-methylated sterol fractions comprise mainly ergosterol and lanosterol plus 4,14-dimethylzymosterol, respectively (22). In control experiments 55 to 63% and 20 to 22% of the NSF radioactivity in both isolates was incorporated into ergosterol and 14α-methylated sterols, respectively. In experiments containing fluconazole to inhibit P-450$_{DM}$, there was a dose-dependent decrease in the proportion of NSF radioactivity in ergosterol and a corresponding increase in the proportion of radioactivity in 14α-methylated sterols for both isolates (Fig. 2). The same phenomena were observed in repeat experiments with cells incubated in HR broth containing [^{14}C]acetic acid as the sterol precursor (Fig. 3). The concentrations of fluconazole required to give 50% inhibition of incorporation (IC_{50}) of mevalonic or acetic acids in ergosterol of lysates and cells were 0.17 ± 0.09 and 7.0 ± 5.65 μM, respectively, for the fluconazole-susceptible strain and 0.20 ± 0.07 and 130.0 ± 22 μM, respectively, for the fluconazole-resistant strain (values are means ± standard deviations of results from two separate exponential-phase cultures [values varied by <10%]). The similar IC_{50}s for susceptible and resistant lysates indicate clearly that fluconazole had a similar potency against the P-450$_{DM}$s from both organisms. However,

TABLE 1. MICs for *C. glabrata* isolates

Compound	MIC (μg/ml)[a]	
	Susceptible	Resistant
Fluconazole	12.5	100
Ketoconazole	0.19	3.1
Itraconazole	0.39	50

[a] The MICs for *C. glabrata* isolates were determined by the broth dilution method.

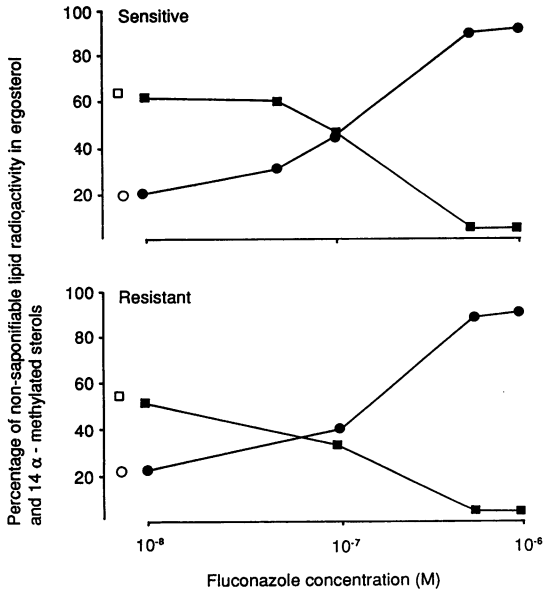

FIG. 2. Effect of fluconazole on incorporation of [^{14}C]mevalonic acid in ergosterol (■) and 14α-methylated sterols (●) of lysates from fluconazole-susceptible and fluconazole-resistant cultures of *C. glabrata*. Values are the means of duplicate determinations which varied by <10%. The control values for ergosterol (□) and 14α-methylated sterols (○) are also shown.

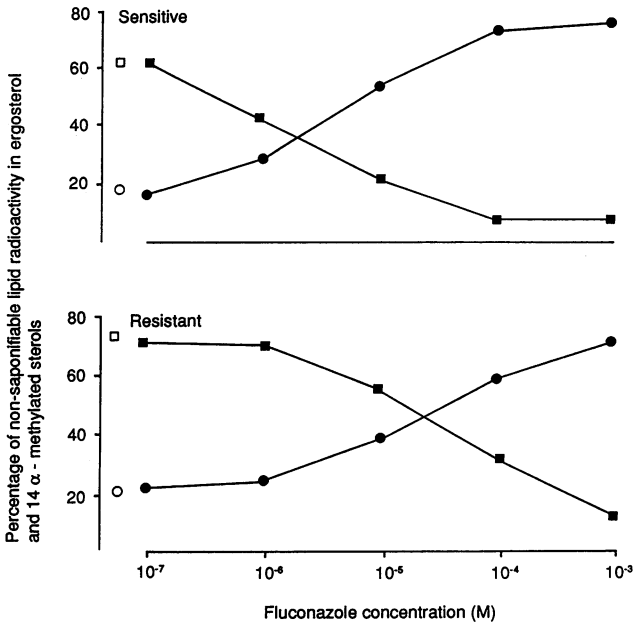

FIG. 3. Effect of fluconazole on incorporation of [^{14}C]acetic acid in ergosterol (■) and 14α-methylated sterols (●) of cells from fluconazole-susceptible and fluconazole-resistant cultures of *C. glabrata*. Values are the means of duplicate determinations which varied by <10%. The control values for ergosterol (□) and 14α-methylated sterols (○) are also shown.

it was much less potent against the enzyme in resistant cells, which is reflected in the larger mean IC_{50} (18.5-fold) compared with that for susceptible cells.

Fluconazole uptake. The results of a typical experiment comparing the uptake of [^{3}H]fluconazole by susceptible and resistant cultures are shown in Fig. 4. The amount of radioactivity incorporated by cells from susceptible cultures was linear during the 30-min incubation period, and the rate of uptake was 0.33 ± 0.02 pmol/min (mean ± standard deviation of experiments on three separate batches of exponential-phase cultures). By contrast, cells from resistant cultures failed to take up the radiolabel. Both susceptible and resistant cells were viable throughout the experiment, as determined by their ability to grow in fresh, drug-free media.

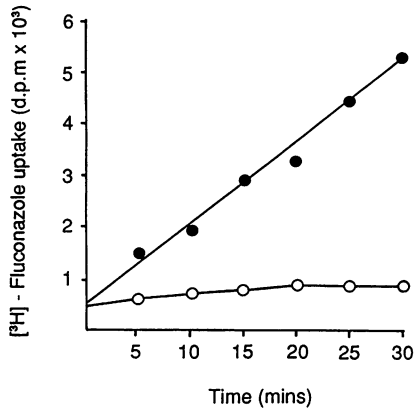

FIG. 4. Uptake of [^{3}H]fluconazole by cells from fluconazole-susceptible (●) and fluconazole-resistant (○) cultures of *C. glabrata*. Values for each time point are the means of triplicate determinations on cells from one culture (values varied by <10%).

DISCUSSION

The emergence of pathogenic yeasts resistant to azole antifungal agents is a rare occurrence, despite their widespread use in many millions of patients during the last 20 years. Furthermore, like other azole-resistant strains of *Candida*, the fluconazole-resistant *C. glabrata* isolate described here was cross-resistant to ketoconazole and itraconazole in vitro.

The RFLP analyses of genomic DNA suggest that the pre- and posttreatment isolates were clonally unrelated. This implies that the resistant organism is not a mutant derived from the susceptible organism but, rather, that it was selected from a mixed population of both organisms by fluconazole treatment.

When cell lysates of susceptible and resistant cultures are incubated with fluconazole, there is a reciprocal relationship between the amounts of ergosterol and 14α-methylated sterols synthesized from [^{14}C]mevalonic acid. This is consistent with the potent inhibition of P-450_{DM} by fluconazole, a phenomenon that has been reported for the enzyme from *C. albicans* both in lysates and in purified preparations (11, 12). Azole resistance in some strains of *C. albicans* and *C. glabrata* appears to arise, either wholly or in part, from the reduced susceptibility of P-450_{DM} to azoles (9, 20, 23). However, this does not apply to the resistant *C. glabrata* isolate described here, since fluconazole potency against its P-450_{DM} in lysates was very similar to that in lysates from susceptible cells. However, when ergosterol biosynthesis was measured in resistant cells by using [^{14}C]acetic acid as the sterol precursor, P-450_{DM} was much less susceptible to fluconazole. This suggests that resistance is due to a permeability barrier to fluconazole rather than to changes in P-450_{DM}.

This hypothesis is supported by the fact that resistant cells were unable to take up [^{3}H]fluconazole. It is interesting in this regard that a number of azole-resistant *C. albicans* strains are impermeable to the triazole [^{14}C]ICI 153,066 (8, 13). The susceptible *C. glabrata* cells described here took up fluconazole at a rate of 1.65 pmol/min/10^9 cells, which compares closely with 2.52 to 3.55 pmol/min/10^9 cells for ICI 153,066 taken up by a range of susceptible *C. albicans* strains (8). It has been shown that different strains of *C. albicans* yeasts and mycelia take up ICI 153,066 at a rate that is proportional to their phospholipid/nonesterified sterol ratio (8, 10). This ratio may influence considerably the physical and biochemical properties of membranes, and sterols are known to reduce the permeabilities of natural and synthetic membranes (2, 3). Although the mechanism(s) of ICI 153,066 or fluconazole uptake is not known, changes in membrane fluidity probably would alter passive diffusion or active transport of the triazoles, depending on their mechanism of uptake. The intention is to investigate whether the proposed relationship between lipid composition and triazole uptake in *C. albicans* extends to *C. glabrata*, and we are currently studying the transport mechanism(s) in both species.

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