

Fluconazole Resistance Due to Energy-Dependent Drug Efflux in *Candida glabrata*

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We report on the mechanism of fluconazole resistance in *Candida glabrata* from a case of infection in which pre- and posttreatment isolates were available for comparison. The resistant, posttreatment isolate was cross-resistant to ketoconazole and itraconazole, in common with other azole-resistant yeasts. Resistance was due to reduced levels of accumulation of [³H]fluconazole rather than to changes at the level of ergosterol biosynthesis. Studies with metabolic or respiratory inhibitors showed that this phenomenon was a consequence of energy-dependent drug efflux, as opposed to a barrier to influx. Since energy-dependent efflux is a characteristic of multidrug resistance in bacteria, yeasts, and mammalian cells, we investigated the possibility that fluconazole resistance is mediated by a multidrug resistance-type mechanism. Benomyl, a substrate for the *Candida albicans* multidrug resistance protein, showed competition with fluconazole for efflux from resistant *C. glabrata* isolates, consistent with a common efflux mechanism for these compounds. By contrast, other standard substrates or inhibitors of multidrug resistance proteins had no effect on fluconazole efflux. In conclusion, we have identified energy-dependent efflux of fluconazole, possibly via a multidrug resistance-type transporter, as the mechanism of resistance to fluconazole in *C. glabrata*.

A growing number of debilitated and immunocompromised patients are at risk of serious fungal infections. These include patients receiving cancer therapies and organ transplants and those infected with the virus that causes AIDS. The latter are particularly susceptible to fungal infections because they are permanently immunocompromised, unlike other groups, in whom immunosuppression is transient. Amphotericin B has been the mainstay of therapy for patients with life-threatening mycoses, although nephrotoxicity and administration by slow intravenous infusion are frequent complications (2). The newer azole class of antifungal agents (fluconazole, itraconazole, and ketoconazole) has also proven to be effective in treating invasive mycoses and represents an important alternative to amphotericin B for some indications (for a review, see reference 28).

The azole antifungal agents work by inhibiting cytochrome P-450-dependent 14 α -sterol demethylase of ergosterol biosynthesis (P-450_{DM}) (for a review, see reference 10). Azole-treated fungi are depleted of ergosterol and accumulate 14 α -methylated sterols which inhibit fungal growth. All of the azoles are fungistatic, as opposed to fungicidal, against *Candida* spp., underlining the importance of the host's immune system for eradicating the infecting organism and achieving a clinical cure. A corollary of this situation is that AIDS patients require indefinite suppressive therapy, and given the widespread use of fluconazole in end-stage AIDS (including patients who have failed ketoconazole or itraconazole therapy), it is not unexpected that fluconazole-resistant *Candida* strains have been isolated from this group of patients (22).

Studies with a number of azole antifungal agents in *Candida albicans*, *Candida glabrata*, and *Saccharomyces cerevisiae* have demonstrated that there are three mechanisms of resistance, viz, changes in P-450_{DM}; changes in Δ^5-6 -sterol desaturase, another enzyme in ergosterol biosynthesis; and permeability resistance (for a review, see reference 11). We recently re-

ported on a case of infection with the haploid yeast *C. glabrata* in which the organism became resistant to fluconazole through reduced drug uptake rather than through changes in ergosterol biosynthesis. Here we show that this phenomenon is due to an energy-dependent efflux mechanism, as opposed to a barrier to influx.

MATERIALS AND METHODS

Materials. Chemicals were of analytical grade and were purchased from Sigma. Benomyl was a gift from Dupont. [³H]fluconazole (specific radioactivity, 714 GBq/mmol) and ³H₂O (specific radioactivity, 37 MBq/g) were purchased from Amersham International and NEN Dupont, respectively.

Strains. *C. glabrata* strains (fluconazole-susceptible strain, Y33.90; fluconazole-resistant strain, Y33.91) were pre- and posttreatment isolates, respectively, from a patient who received fluconazole therapy (12). The azole MICs for these organisms were determined by the broth dilution method (20). The isolates were maintained in freeze-dried ampoules and were subcultured onto Sabouraud dextrose agar plates before use.

Measurement of fluconazole uptake. Fluconazole uptake was measured by a filter-based assay adapted from the method described previously (12). Cultures were grown in Sabouraud dextrose broth to a density of 10⁸ cells per ml. The cells were centrifuged and the pellet was resuspended in phosphate-buffered saline (PBS; pH 7.5) to the original cell density. Aliquots (60 ml) of the cell suspension were transferred to 125-ml flasks, and the following compounds were added separately in 0.12 ml of ethanol or dimethyl sulfoxide: carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP), antimycin A, cycloheximide, colchicine, verapamil, benomyl, methotrexate, nitroquinoline oxide, aminotriazole (final concentration, 100 μ M), oligomycin, daunomycin, ethidium bromide, reserpine (final concentration, 10 μ M), and sodium azide (final concentration, 1 mM). [³H]fluconazole (12 μ l) and unlabelled fluconazole (270 μ l of a 20- μ M solution in PBS) were added to give a final fluconazole concentration and specific radioactivity of 100 nM and 7.4 kBq/ml, respectively. The flasks were incubated at 37°C with shaking at 170 rpm. At various times, triplicate samples of 3 ml each were removed and filtered in a Millipore vacuum manifold with Whatman GF/C filters which had been pre-soaked in 100 μ M unlabelled fluconazole. The filters were washed four times with 4 ml of PBS containing 100 μ M unlabelled fluconazole and were transferred to 20-ml scintillation vials. The filters were dried at 37°C for 60 mins before Ecocint A scintillation fluid (10 ml) was added. The vials were capped and were left at room temperature overnight before measurement of their radioactivity in a Wallac 1410 scintillation counter. Control experiments with heat-killed cells and blank assays without cells were done to establish the amount of drug binding to cells and filters. These amounts were reproducible and did not exceed 10% of the incorporated radioactivity.

Measurement of intracellular volume. The intracellular volumes of the sus-

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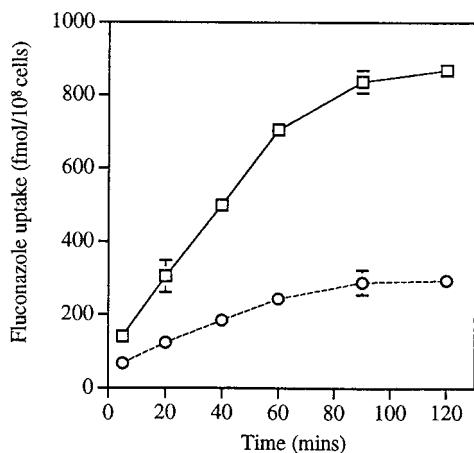


FIG. 1. Uptake of [³H]fluconazole by cells from fluconazole-susceptible (□) and fluconazole-resistant (○) cultures of *C. glabrata*. Values for each time point are the means \pm standard deviations of triplicate determinations with cells from one culture.

ceptible and resistant cells were estimated by measuring ³H₂O uptake by the method described by Boiron et al. (4).

RESULTS

Azole susceptibilities of *C. glabrata* isolates. The fluconazole, ketoconazole, and itraconazole MICs were determined to be 12.5, 0.19, and 0.39 $\mu\text{g} \cdot \text{ml}^{-1}$, respectively, for strain Y33.90 and 100, 3.1, and 50 $\mu\text{g} \cdot \text{ml}^{-1}$, respectively, for strain Y33.91. Thus, strain Y33.91 is some 8-fold less susceptible to fluconazole, 16-fold less susceptible to ketoconazole, and 128-fold less susceptible to itraconazole than strain Y33.90.

Fluconazole uptake. The results of experiments comparing the uptake of [³H]fluconazole by cultures of susceptible and resistant strains are shown in Fig. 1. Cultures of the susceptible strain took up fluconazole at a rate of 12 fmol/min for approximately 60 min. Thereafter, the rate of fluconazole uptake steadily decreased and reached a plateau when the cells had accumulated 869 fmol of drug. The results of the corresponding study with resistant cells show that they were less able to take up drug than susceptible cells (294 versus 869 fmol after 120 min of incubation). The intracellular volumes of susceptible and resistant cells were estimated by measuring ³H₂O accumulation, thereby enabling the calculation of intracellular fluconazole concentrations on the basis of the amounts of radiolabelled drug taken up by the cells. Thus, after 80 min of incubation in the standard assay described above, the intracellular concentration of fluconazole in susceptible cells was 2.5-fold greater than that in the extracellular incubation medium (260 ± 9.42 versus 107 ± 1.07 nM, respectively; $n = 3$). By contrast, the intracellular concentration of fluconazole in the resistant cells was 0.5-fold lower than that in the extracellular incubation medium (57 ± 3.23 versus 108.2 ± 1.27 nM, respectively; $n = 3$) and, therefore, 4.5-fold lower than the intracellular concentration in susceptible cells. This is consistent with an efficient mechanism of permeability resistance.

Effect of respiratory and metabolic inhibitors on fluconazole uptake. The effects of different classes of respiratory inhibitors (uncouplers, CCCP and FCCP; electron transport chain inhibitors, sodium azide and antimycin A; and mitochondrial ATPase inhibitor, oligomycin) on [³H]fluconazole uptake were examined by adding these compounds to the standard uptake assay. The results are summarized in Fig. 2 and indicate that antimycin A and CCCP caused a small increase in the uptake

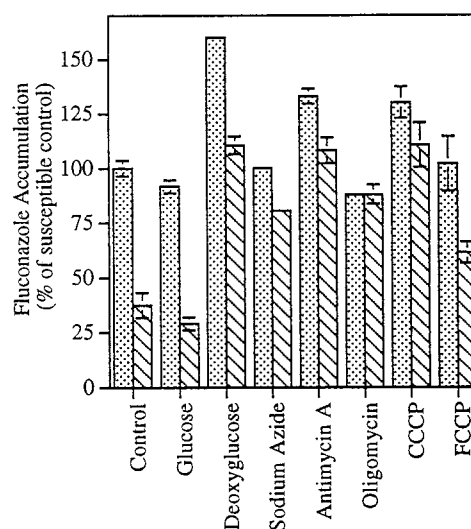


FIG. 2. Effect of glucose or respiratory inhibitors on [³H]fluconazole uptake by cells from fluconazole-susceptible (□) and fluconazole-resistant (■) cultures of *C. glabrata* after 80 min of incubation in the standard uptake assay. Values are means \pm standard deviations of triplicate determinations with cells from one culture.

of fluconazole by susceptible cells, whereas the other inhibitors had no effect on uptake. Repeat experiments with cells from cultures of the resistant strain produced quite different results, since all of the inhibitors markedly increased the uptake of fluconazole by resistant cells. In fact, the amount of fluconazole taken up by these cells was comparable to that taken up by the susceptible cells, indicating that the permeability resistance mechanism is energy dependent. The same phenomenon was observed when the respiratory inhibitors were replaced by the metabolic inhibitor 2-deoxyglucose in the uptake assay. This emphasizes further the dependence of permeability resistance on the energy status of the resistant cells. In some experiments, cells were supplied with glucose (1% [wt/vol]) via the assay medium so as to maintain, rather than deplete, their energy status. In contrast to the respiratory and metabolic inhibitors, glucose caused a small decrease in the uptake of fluconazole by both susceptible and resistant cells. Taken together, these data demonstrate that permeability resistance to fluconazole in *C. glabrata* is due to an energy-dependent efflux mechanism rather than to a barrier to influx. It is interesting in this regard that energy-dependent efflux mechanisms are known to confer multidrug resistance (MDR) in tumor cells, antibiotic resistance in some bacteria, and resistance to a wide range of cytotoxic compounds in the yeasts *S. cerevisiae* and *C. albicans* (3, 8, 9).

Effect of known substrates and inhibitors of MDR proteins on fluconazole uptake. In an attempt to characterize further the energy-dependent efflux mechanism for fluconazole in *C. glabrata*, substrates of known efflux pumps (i.e., MDR proteins) were profiled in the standard [³H]fluconazole uptake assay. The rationale for this approach is that an efflux mechanism shared by fluconazole and the compound under investigation should increase the level of accumulation of radiolabelled fluconazole through competition for efflux between the two substrates. In line with this, the following compounds were chosen for study: colchicine and daunomycin, substrates for mammalian MDR proteins (1, 13, 21, 26); ethidium bromide, a substrate for bacterial MDR proteins (16, 27); aminotriazole, a substrate for *S. cerevisiae* MDR proteins (15); benomyl, a

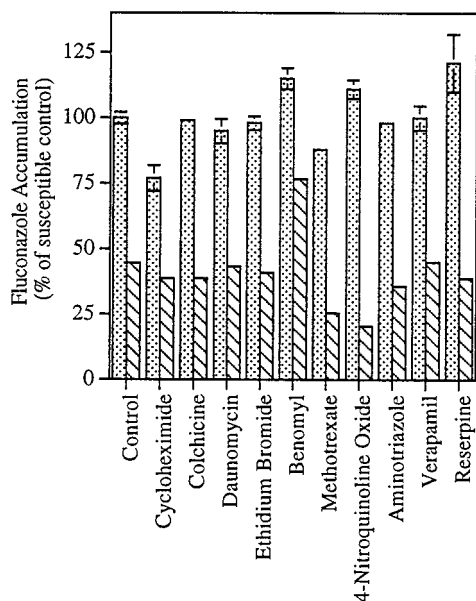


FIG. 3. Effects of MDR protein substrates or inhibitors on [3 H]fluconazole uptake by cells from fluconazole-susceptible (□) and fluconazole-resistant (▨) cultures of *C. glabrata* after 80 min of incubation in the standard uptake assay; the assay was extended to 180 min for verapamil. Values are means \pm standard deviations of triplicate determinations with cells from one culture.

substrate for *C. albicans* MDR proteins; and cycloheximide and nitroquinoline oxide, substrates for both *S. cerevisiae* and *C. albicans* MDR proteins (3, 24). Interestingly, benomyl was the only compound that caused an increase in fluconazole uptake by *C. glabrata*, and resistant cells were significantly more active than susceptible cells in this respect (Fig. 3). Nitroquinoline oxide reduced fluconazole uptake in both susceptible and resistant cells.

Another way of probing the fluconazole efflux mechanism in *C. glabrata* is to test standard inhibitors of MDR proteins, as opposed to substrates. Both bacterial and mammalian MDR proteins have been shown to be inhibited by reserpine and verapamil (5, 18, 19, 25), and consequently, they were included in the [3 H]fluconazole uptake assay. In contrast to benomyl and nitroquinoline oxide, neither reserpine nor verapamil had any significant effect on fluconazole uptake in either susceptible or resistant cells, and the data are included in Fig. 3 for comparison.

DISCUSSION

Studies with *Candida* spp. and *S. cerevisiae* have demonstrated that there are several mechanisms by which these organisms can become resistant to azole antifungal agents in vitro (11). They depend on the azole antifungal agent under investigation, the organism, and the nature of the growth medium on which the organism is grown. By contrast, there would appear to be only three mechanisms of resistance in the small number of clinical isolates that have been examined to date (11). These are overexpression of the target enzyme P-450_{DM}, thereby reducing azole binding, or lesions in Δ^{5-6} -sterol desaturase which compensate for azole inhibition of P-450_{DM} through changes in sterol composition; or a reduced level of accumulation of drug. Furthermore, each mechanism confers cross-resistance to all of the commercially available azole antifungal agents. Consistent with this, the present fluconazole-resistant *C. glabrata* isolate shows greatly reduced susceptibil-

ities to ketoconazole and itraconazole (12). By comparing the inhibition of ergosterol biosynthesis in resistant cells with that in susceptible cells, we have demonstrated previously that resistance is unrelated to changes in P-450_{DM} or Δ^{5-6} -sterol desaturase (12). Rather, it arises from the inability of resistant cells to take up [3 H]fluconazole, in contrast to the case for susceptible cells, which readily accumulate the radiolabel.

In the present study, treatment of resistant cells with respiratory or metabolic inhibitors caused them to accumulate fluconazole to the same extent as susceptible cells, indicating that permeability resistance is due to an energy-dependent efflux mechanism. Interestingly, some of the inhibitors caused a small but similar effect in susceptible cells, suggesting that they may also possess an energy-dependent efflux mechanism, albeit at much lower levels than resistant cells. Further evidence in support of this hypothesis is the fact that glucose, a substrate for glycolysis and respiration, produced a decrease in fluconazole accumulation in both susceptible and resistant cells. A similar energy-dependent efflux mechanism has been described in the plant fungal pathogens *Penicillium italicum* and *Nectria haematococca* for fenarimol, a pyrimidine ergosterol biosynthesis inhibitor (6, 7, 14). Uptake was by passive diffusion, and resistant strains showed a greater rate of efflux than susceptible ones. It is interesting in this respect that energy-dependent efflux mechanisms confer resistance to several different classes of compounds in bacteria, yeasts, and mammalian cells (9). MDR in human tumor cells has been the subject of intensive investigation and is mediated by a single membrane-bound transporter protein (P-glycoprotein) (8, 13). Much less is known about the corresponding MDR protein transporters of bacteria and yeasts, but they would appear to be different from one another and from the mammalian P-glycoprotein when their biochemical properties (e.g., energized by ATP hydrolysis versus proton motive force) and substrate specificities are compared. In order to determine whether fluconazole efflux in *C. glabrata* is mediated by an MDR protein, we looked for competition between fluconazole and the substrates or inhibitors of previously characterized MDR protein transporters. The only compound to compete with fluconazole efflux was benomyl, a substrate for *C. albicans* MDR protein (3). This implies that benomyl is able to penetrate *C. glabrata* and interact with a putative fluconazole efflux transporter. The mammalian MDR protein inhibitor verapamil is also taken up by *C. glabrata* on the basis of [3 H]desmethoxyverapamil (a derivative of verapamil) transport studies (data not shown). This, together with verapamil's lack of activity against fluconazole efflux, would suggest that it has a low affinity for the efflux transporter. Unlike verapamil, colchicine (another mammalian MDR protein inhibitor) is unable to penetrate *C. glabrata* when [3 H]colchicine uptake is measured (data not shown). This explains why colchicine had no effect on fluconazole efflux, but it does not rule out the possibility that colchicine is an inhibitor of the efflux transporter. This phenomenon may also apply to the other MDR protein inhibitors or substrates tested in the present study, and measurement of their levels of uptake and intracellular concentrations in *C. glabrata* will be required to test this hypothesis. The rapid and marked reduction in fluconazole uptake by susceptible and resistant cells caused by nitroquinoline oxide was unexpected and is difficult to explain. In addition to being a substrate for some MDR proteins, this compound is a mutagen, and it is difficult to reconcile how this activity would affect fluconazole transport during the short duration of the experiment. Instead, it may reflect nitroquinoline oxide binding to membranes, thereby adversely affecting fluconazole influx in both susceptible and resistant cells. Another way of characterizing the fluconazole efflux mechanism is

by a rigorous kinetic analysis in purified plasma membrane preparations or by purifying the putative transporter protein and reconstituting it in model membrane systems. These studies are in progress, together with those aimed at identifying the gene encoding the putative transporter protein. Leonard et al. (17) showed that mutation of the *Saccharomyces* MDR gene, *PDR5*, resulted in a decrease in the efflux of chloramphenicol from the cells. Similar studies should be possible with the cloned fluconazole resistance gene.

Reduced permeability to azoles as a mechanism of resistance in *C. albicans* has been studied intensively in strains isolated from patients with chronic mucocutaneous candidiasis who failed ketoconazole therapy (strains AD and KB). Unlike azole-susceptible strains, *C. albicans* AD and KB failed to take up the radiolabelled experimental triazole ICI 153,066 (23). It has been suggested that this could reflect changes in the phospholipid/nonesterified sterol ratio in the membranes of resistant cells (11). This ratio is known to influence the structures and functions of membranes, and sterols reduce the permeabilities of both natural and model membranes. Although the mechanism of permeability resistance to ICI 153,066 is not known, the marked changes in the lipid compositions of the membranes of resistant cells probably would alter passive diffusion or active transport (influx and efflux mechanisms), depending on the mechanism of uptake. We are investigating whether the relationship between membrane lipid composition and triazole transport in *C. albicans* extends to *C. glabrata* and the influence that this may have on fluconazole efflux in both resistant and susceptible cells. It would also be interesting to investigate whether the efflux mechanism is expressed in *C. albicans* and other fungal pathogens.

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