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Correlation between Rhodamine 123 Accumulation and Azole Sensitivity in *Candida* Species: Possible Role for Drug Efflux in Drug Resistance

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A wide variety of prokaryotic and eukaryotic cells exhibit a multidrug resistance (MDR) phenotype, indicating that resistance to potentially toxic compounds is mediated by their active efflux from the cell. We have sought to determine whether resistance to azoles in some strains of *Candida* species may be due in part to active drug efflux. Rhodamine 123 (Rh123) is a fluorescent compound that is transported by a wide variety of MDR cell types. We have shown that certain azole-resistant strains of *Candida albicans*, *C. glabrata*, and *C. krusei* accumulate less Rh123 than azole-susceptible ones. In *C. albicans*, Rh123 accumulation was growth phase and temperature dependent and was increased by proton uncouplers and by reserpine, an MDR modulator. This is consistent with an energy-dependent efflux mechanism for Rh123, mediated by an MDR transporter. In *C. glabrata*, but not in *C. albicans*, there was competition between Rh123 and fluconazole for efflux. Thus, in *C. glabrata*, Rh123 and fluconazole appear to be transported via a common MDR-like transporter, whereas in *C. albicans*, the Rh123 transporter does not appear to transport azoles.

Candida albicans is an asexual diploid fungus which is an opportunistic pathogen in humans. It can cause a variety of infections, ranging from superficial mycoses to life-threatening systemic infections commonly seen in immunocompromised patients (37). Treatment of systemic *Candida* infections is difficult and is limited to three classes of antifungal agents: the polyene macrolide antibiotic amphotericin B, the substituted pyrimidine flucytosine (5FC), and the N-substituted imidazole and triazole derivatives. Polyenes bind to ergosterol in the fungal cell membrane. Consequently, they do not have to enter the fungal cell to be effective and clinical resistance is rare, although it can be induced under laboratory conditions (46). The rate of 5FC resistance in natural populations of *C. albicans* is relatively high, reflecting, in part, a natural heterozygosity at genetic loci encoding 5FC resistance (52). This, in turn, hampers 5FC therapy of candidiasis, in which resistance can occur during relatively short courses of treatment, irrespective of the immune status of the patient (37). Resistance to azoles in *C. albicans* appears to be associated mainly with long-term therapy in severely immunocompromised patients, such as those with late-stage AIDS or chronic mucocutaneous candidiasis (for a review, see reference 43). By contrast, azole resistance is reported rarely for nonimmunocompromised patients and patients with transient immunosuppression, such as cancer patients, organ transplant recipients, and patients in the early stages of AIDS. Several reports of cross-resistance to different azole antifungal agents have been published (45, 53).

The fungistatic action of the azoles is the result of the disruption of a complex multistep pathway in ergosterol biosynthesis. Thus, there are a variety of mechanisms by which resistance to azoles may occur (22, 51). Changes in cytochrome P-450-dependent 14α-sterol demethylase (P-450_{DM}), the target for azole action, or in other enzymes involved in ergosterol biosynthesis ($\Delta^{5,6}$ sterol desaturase) can render the cells less susceptible to azoles (22, 51). Resistance to antifungal drugs may also relate to changes in the permeability of the cell. Such changes have been associated with alterations in the sterol content that affect the fluidity (and hence the permeability) of the membrane (23, 51). Active efflux mechanisms can also reduce the permeability of the cell, and Crombie et al. (14) have reported recently that fluconazole resistance in *C. glabrata* is due to energy-dependent efflux of the drug by a multidrug resistance (MDR)-type mechanism.

In mammalian cells, the phenomenon of MDR occurs when exposure to a single toxic agent induces resistance to a wide range of structurally and functionally unrelated drugs. The common phenotype is mediated by the presence of a membrane-located pump, P glycoprotein (P-gp), which facilitates the energy-dependent transport of drugs out of the cell (1, 13, 20). On the basis of amino acid sequence homology, P-gp is related to a family of transport proteins, the ABC (ATP-binding cassette) transporters (17, 31, 39). These proteins contain a common ATP-binding motif and utilize the energy from ATP hydrolysis to transport a wide variety of substrates.

The MDR phenotype has been studied in a diverse range of organisms, from archaebacteria (33) to humans (30); however, the phenotype does not appear to be mediated by a P-gp-like pump in all organisms. The MDR phenotype has been detected in *Bacillus subtilis* (34), *Escherichia coli* (32), and *Staphylococcus aureus* (35), but in these organisms, efflux is dependent on a proton gradient across the cell membrane rather than ATP hydrolysis. These proteins are members of the major facilitator superfamily (MFS) of transport proteins, and, unlike the ABC transporters, the MFS proteins do not share any significant primary sequence similarity with each other (5). For the yeast *Saccharomyces cerevisiae*, a number of genes which are believed to be involved in MDR have been cloned (4). More than seven *PDR* (pleiotropic drug resistance) genes in *S. cerevisiae* have since been identified, many of which are believed to encode transcriptional regulators (15). However, *PDR5* is thought to code for an ABC transporter and may be of importance in resistance to antifungal agents (6, 39). Transporters of the MFS type have also been identified in *S. cerevi-*

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siae (5). Recently, a gene homologous to *PDR5*, designated *CDR1*, has been isolated from *C. albicans* and shown to be involved in the MDR phenotype (42). A gene encoding a protein of the MFS has also been cloned from *C. albicans* (8). Therefore, fungi have a range of efflux transport proteins that may be involved in detoxifying cells that are exposed to potentially harmful molecules.

Although the azoles have a common mode of action, their structures and aqueous solubilities are diverse. We therefore investigated whether permeability resistance to azoles in *C. albicans* may be due to MDR-mediated efflux. Accumulation of rhodamine 123 (Rh123), a known substrate for a wide diversity of cells exhibiting the MDR phenotype (33, 34), was used as an indicator of putative drug efflux-transporter activity in azole-sensitive and azole-resistant strains.

MATERIALS AND METHODS

Materials. Fluconazole was synthesized by Pfizer Central Research, Sandwich, Kent, United Kingdom. ICI 153,066 (7) was supplied by Zeneca Pharmaceuticals, Alderly Park, Cheshire, United Kingdom. Ketoconazole and itraconazole were extracted from commercial preparations. Methotrexate was a kind gift from R. J. Howard (Du Pont Agricultural Products, Wilmington, Del.). [^{14}C]leucine (specific radioactivity, 11.4 GBq/mmol) and [^{3}H]fluconazole (specific activity, 714 GBq/mmol) were from Amersham. All other chemicals were supplied by Sigma (Poole, Dorset, United Kingdom) and were of analytical grade.

Strains and culture conditions. *C. albicans* 3153 and 3302 (44) are azole-susceptible and azole-resistant isolates, respectively, and were obtained from the National Collection of Pathogenic Fungi. Strain 3302 (also known as strain AD [23, 45]) was isolated originally from a 9-year-old girl with chronic mucocutaneous candidiasis (26). *C. glabrata* Y33.90 (azole susceptible) and Y33.91 (azole resistant) are pre- and posttreatment isolates, respectively, from a patient who received fluconazole therapy (24). *C. krusei* CK5 and KM3 were kindly supplied by D. Kerridge, Department of Biochemistry, University of Cambridge. Strain KM3 has increased resistance to fluconazole and was obtained by selection on fluconazole-containing agar plates (51a). All *Candida* species used were maintained on Sabouraud dextrose agar plates at 4°C. Overnight cultures of single colonies were grown in 10 ml of Sabouraud dextrose liquid medium (4% [wt/vol] glucose, 1% [wt/vol] mycological peptone) (SDB) in Universal bottles. Aliquots of these cultures were used to inoculate 500-ml Erlenmeyer flasks containing 100 ml of SD medium (48), and cultures were grown to mid-log phase at an optical density (OD) at 600 nm (OD_{600}) of precisely 0.60 ± 0.1 before cells were harvested. *C. albicans* strains were grown in the yeast form only.

Rh123 accumulation in yeast cells. An assay, modified from that described by Efferth et al. (16), was devised to enable the retention of Rh123 in *Candida* yeast cells to be measured. Rh123 is a substrate for MDR efflux transporters from a diversity of species, from archaebacteria (33) to humans (12). Rh123 is believed to enter cells by diffusion (9). Cells from the mid-log phase of growth were pelleted at 450 × *g* for 3 min and then resuspended in 0.01 M phosphate-buffered saline (PBS), pH 7.4. Addition of 10 mM glucose had no effect on Rh123 accumulation during the 30-min assay period (data not shown). The cells were incubated in a shaking water bath (200 rpm) at 37°C, and Rh123 was added to the cultures to a final concentration of 13 μM. The cells were incubated in Rh123 for various lengths of time, and then 2-ml samples were withdrawn, filtered on Whatman GF/C filters by using a Millipore vacuum manifold, and briefly washed twice with PBS. After external Rh123 was removed, the fluorescence and OD of the cells were determined spectrophotometrically. Fluorescence was measured with a FluoroskanII mictotiter plate fluorimeter (Flow Laboratories, Richmond, Hertfordshire, United Kingdom) with excitation and emission wavelengths of 485 and 538 nm, respectively. In the calculation of accumulation ratios for Rh123 in the cells, the external Rh123 concentration was a known value and was checked against a calibration curve. The internal concentration was calculated by determining the Rh123 fluorescence of washed cells. The OD_{600} of the cells was measured. Rh123 accumulation was expressed as arbitrary fluorescence units per OD unit (specific fluorescence). Twelve replicate samples were taken in triplicate independent experiments. The results are given as means ± standard errors (SE).

In some experiments, the effects of the addition of various antifungal compounds, metabolic inhibitors, and MDR modulators on Rh123 accumulation were determined. Fluconazole was dissolved in distilled H_2O; ketoconazole, CCCP (carbonyl cyanide *m*-chlorophenylhydrazone), antimycin A, verapamil, cycloheximide, colchicine, and daunomycin were dissolved in 100% ethanol; ICI 153,066 was dissolved in dimethyl sulfoxide (DMSO); reserpine was dissolved in chloroform; and methotrexate was dissolved in 0.1 M NaOH. Controls included addition of appropriate amounts of solvent to the assay in the absence of the drug.

Fluconazole accumulation in yeast cells. [^{3}H]fluconazole uptake was measured by using a filter-based assay similar to that used for the Rh123 assay. Cultures were grown in SDB to a density of 10^8 cells per ml. The cells were centrifuged, and the pellet was resuspended in PBS to the original cell density. Aliquots (60 ml) of the cell suspension were transferred to 125-ml flasks, and Rh123 was added. [^{3}H]fluconazole (12 μl) and unlabelled fluconazole (270 μl of a 20 μM solution in PBS) were added to give a final fluconazole concentration and specific radioactivity of 100 nM and 7.4 GBq/ml, respectively. The flasks were incubated at 37°C with shaking at 170 rpm. Fluconazole accumulation was shown to reach equilibrium after 80 min (data not shown), and the cells were therefore analyzed at this time. Triplicate samples of 3 ml were removed and filtered in a Millipore vacuum manifold with Whatman GF/C filters which had been presoaked in 100 μM unlabelled fluconazole. The filters were washed four times with 4 ml of PBS containing 100 μM unlabelled fluconazole and transferred to 20-ml scintillation vials. The filters were dried at 37°C for 60 min, and then Ecoscint A scintillation fluid (10 ml) was added. The vials were capped and 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 performed to establish the amounts of drug binding to the cells and filters. These amounts were reproducible and did not exceed 10% of the incorporated radioactivity.

Respiration measurements. To determine the effects of CCCP, antimycin A, and other metabolic inhibitors and uncouplers on *C. albicans* cells, oxygen consumption was measured with a Clark oxygen electrode. Cells grown to an OD_{600} of 0.60 were resuspended in PBS with or without glucose at 37°C, and a base rate of oxygen consumption was established over 3 min. Respiratory inhibitors were added, and the effect on oxygen consumption was determined.

Measurement of ATP pools. To determine the effects of metabolic inhibitors on *C. albicans* cells, endogenous ATP was measured by using a bioluminescent ATP assay kit (Sigma). The assay was based on the conversion of luciferin to light by firefly luciferase in the presence of ATP. Light emitted was measured at 490 nm by using 1251 automated luminometer (LKB, Luckfield, East Sussex, United Kingdom) and was proportional to the ATP present. Controls were performed to determine the optimal recoveries of ATP from yeast cells. *C. albicans* cells were disrupted by being vortexed for 3 min with glass beads or permeabilized by being heated at 100°C for 5 min or by being treated with DMSO. Treatment with DMSO and physical disruption with glass beads were found to give the highest ATP yields from identical samples (data not shown). Cell debris was removed by centrifugation in a microcentrifuge for 3 min. Supernatants were stored at −20°C. ATP pools were measured according to the manufacturers' instructions, and results were expressed as moles of ATP per *C. albicans* yeast cell.

Fluorescence microscopy. The accumulation of Rh123 in *C. albicans* yeast cells was confirmed by fluorescence microscopy. Cells were grown to an OD_{600} of 0.60 at 37°C and harvested as described above. Cells were incubated with Rh123 and examined after 0, 5, 10, 15, 20, and 30 min. The cells were observed with an Olympus BH2 fluorescence microscope fitted with GB filters.

Measurement of transport of amino acids. Cells treated with metabolic inhibitors were assessed for their ability to transport amino acids. Uptake of [^{14}C]leucine was determined for cells grown to an OD_{600} of 0.60 as described above. [^{14}C]L-leucine and unlabelled leucine were added to give a final leucine concentration and specific radioactivity of 10 μM and 951 kBq/μmol, respectively. Triplicate samples (100 μl) were removed, washed by filtration, and dried and the radioactivity was counted as described above.

MIC tests. The sensitivities of *Candida* strains to various azoles were tested by broth macrodilution in high-resolution solid medium (41). The MIC was taken to be the lowest drug concentration to prevent visible growth. In the absence of any agreed definitions and cutoff points to distinguish susceptible and resistant strains, we have used the term "resistant" to indicate a strain for which the MIC is markedly increased compared with those for other, more sensitive strains examined under identical conditions (41). Such strains might also be legitimately considered less susceptible to inhibition by azole antifungal agents.

RESULTS

Characterization of azole susceptibilities of strains. The relative MICs for the *Candida* strains used were determined by using fluconazole and other azole antifungal agents (Table 1). These results show that the MIC for the clinical isolate *C. albicans* 3302, which had proved unresponsive to ketoconazole therapy in vivo is 33-fold higher than that for *C. albicans* 3153. Strain 3302 also has greatly reduced susceptibilities to itraconazole and fluconazole, with 520- and 32-fold increases in MICs, respectively. The *C. glabrata* strains used in this study are pre- and posttreatment isolates from a patient treated with fluconazole. Strain Y33.91 became resistant to fluconazole during therapy and is some eightfold less susceptible to fluconazole than strain Y33.90. Like *C. albicans* 3302, *C. glabrata* Y33.91 is also significantly less susceptible to ketoconazole and itraconazole, with 16- and 128-fold increases in MICs, respectively. With both resistant organisms, the greatest increase in MIC

TABLE 1. MICs for *Candida* strains

Organism	MIC (μg/ml)[a]		
	Fluconazole	Ketoconazole	Itraconazole
C. albicans			
3153	0.78	0.012	0.003
3302	25	0.39	1.56
C. glabrata			
Y33.90	12.5	0.19	0.39
Y33.91	100	3.1	50
C. krusei			
CK5	25	0.19	0.04
KM3	100	0.39	0.19

[a] Determined by using the broth dilution method (41).

was for itraconazole. The MIC of fluconazole for *C. krusei* KM3, which was selected for fluconazole resistance in vitro, is increased fourfold relative to that for the parental strain, CK5, and the MICs of ketoconazole and itraconazole for the strain are increased two- and fivefold, respectively.

Studies with the triazole derivative ICI 153,066 have shown that resistance in *C. albicans* 3302 is due to reduced drug accumulation, rather than to changes in the target enzyme, P-450_{DM} (45). It is not known if this phenotype is mediated by drug efflux. Furthermore, the same phenomenon has been reported for fluconazole in *C. glabrata* Y33.91, for which the decreased permeability was shown to be a consequence of energy-dependent drug efflux (14).

Accumulation of Rh123 by *Candida* cells. The accumulation of Rh123 in *Candida* yeast cells was used to explore the relationship between putative drug efflux mechanisms and resistance to azole antifungal agents. Rh123 accumulation reached equilibrium after 20 min (data not shown), and consequently, cells were analyzed routinely after 30 min of incubation. Rh123 accumulation was proportional to the concentration of Rh123 in the medium, as determined by varying the Rh123 concentration from 1.3 to 130 μM. Cells of *C. albicans* 3153 that were fixed in 5% formalin for 10 min and resuspended in PBS had a threefold-higher level of specific Rh123 fluorescence and did not exclude Rh123 against a concentration gradient (Table 2). Formalin-treated cells were dead, as assessed by viable counts on Sabouraud dextrose agar and by the capacity of the fixed cells for active transport of [^{14}C]leucine (Table 2). These results suggest that the specific fluorescence of Rh123-treated cells is influenced by cell viability.

In mammalian systems, Rh123 has been shown to accumulate in mitochondria (29). Rh123-treated *C. albicans* yeast cells stained diffusely and homogeneously throughout the cytoplasm. Experiments in which cells were plasmolyzed by using 2 M sorbitol so that the cytoplasmic membrane was withdrawn from the cell walls of some cells suggested that Rh123 staining was not associated with the cell wall.

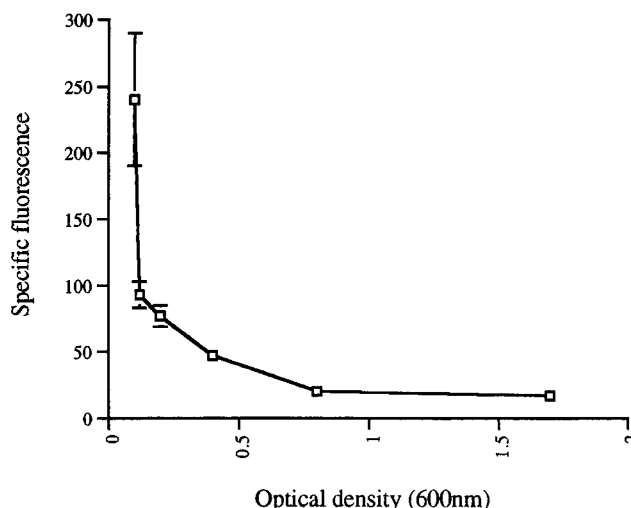

FIG. 1. Rh123 accumulation presented as specific fluorescence measurements of cells harvested at intervals during batch culture of *C. albicans* 3302 in SDB at 37°C. Error bars show SE (n = 12). Errors of <3% are not shown.

Rh123 accumulation in *C. albicans* 3302 (Fig. 1) and 3153 (not shown) was influenced by the growth phase of the cells. Maximum accumulation occurred during the lag phase and early exponential phase of growth and decreased steadily during late-logarithmic-phase growth and during the stationary phase. Because of the growth phase dependency, experiments were standardized by using cells in the mid-log phase of growth at an OD_{600} of 0.60 ± 0.1.

Rh123 accumulation in *C. albicans* cells that were resuspended in PBS with and without 10 mM glucose was measured. The presence or absence of glucose had no effect on Rh123 accumulation or on the cellular ATP pool concentration [means ± SE, $(65 \pm 15) \times 10^{-2}$ and $(75 \pm 19) \times 10^{-2}$ fmol of ATP per cell, respectively; n = 12] over the 30-min period of the Rh123 assay. Subsequent assays were performed at 37°C with washed, mid-exponential-phase cells that were resuspended in PBS alone. Cell metabolism is predominantly fermentative when cells are harvested at this stage of growth and under the conditions employed to measure Rh123 accumulation (2, 3).

Accumulation of Rh123 by azole-susceptible and azole-resistant cells. Rh123 accumulation in the azole-resistant and the azole-susceptible strains of *C. albicans* and *C. glabrata* was compared for mid-log-phase cells at 37°C (Fig. 2). *C. albicans* 3153 accumulated fourfold more Rh123 (mean specific fluorescence ± SE, 224 ± 3; n = 48) than *C. albicans* 3302 (55 ± 1; n = 48). No difference in Rh123 accumulation was seen between strain 3153 (mean specific fluorescence ± SE, 221 ± 25; n = 12) and strain 3302 (221 ± 21; n = 12) when the cells

TABLE 2. Accumulation of Rh123 and leucine in live and dead cells of *C. albicans*

Strain	Treatment	Rh123 specific fluorescence[a]	Accumulation ratio[b]	[^{14}C]leucine uptake (cpm/10^6 cells/min)[c]
3153 (sensitive)	None (control)	220 ± 23	0.36	1,172 ± 14
	Formalin	661 ± 69	1.14	83 ± 9.5
3302 (resistant)	None (control)	72 ± 14	0.12	1,356 ± 21
	Formalin	603 ± 54	0.98	90 ± 9.6

[a] Specific fluorescence measurements were determined as described in Materials and Methods and are means ± standard errors (n = 12).
[b] Defined as the ratio of the internal Rh123 concentration to the external Rh123 concentration.
[c] Measured for cells at 37°C. Values are means ± standard errors (n = 5).

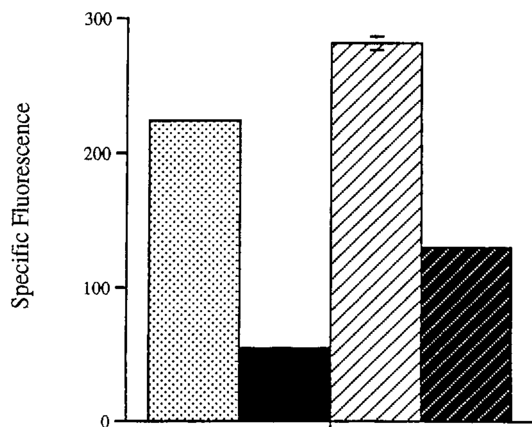

FIG. 2. Rh123 accumulation in azole-susceptible and azole-resistant strains of *C. albicans* and *C. glabrata* from the mid-logarithmic phase of growth at 37°C. Error bars show SE (n = 12 for *C. albicans* and n = 3 for *C. glabrata*). Errors of <3% are not shown. Three replicate experiments were performed for *C. albicans*, and a single experiment was performed for *C. glabrata*. ▧, *C. albicans* 3153 (azole susceptible); ■, *C. albicans* 3302 (azole resistant); ▨, *C. glabrata* Y33.90 (azole susceptible); ▨, *C. glabrata* Y33.91 (azole resistant).

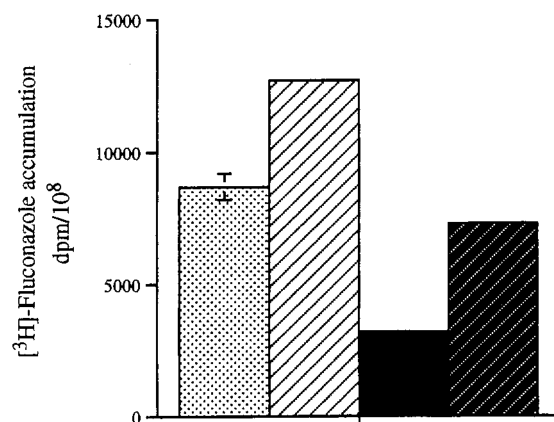

FIG. 3. [^{3}H]fluconazole accumulation by azole-sensitive and azole-resistant strains of *C. glabrata* in the presence and absence of 130 μM Rh123. Error bars show SE (n = 3) from a single experiment. Errors of <3% are not shown. ▧, Y33.90 (azole susceptible); ▨, Y33.90 plus Rh123; ■, Y33.91 (azole resistant); ▨, Y33.91 plus Rh123.

were grown at 25°C. Similarly, *C. glabrata* Y33.90 accumulated twofold more Rh123 than the resistant strain Y33.91 (282 ± 5 [n = 3] versus 130 ± 4 [n = 3]).

We reasoned that if azole antifungal agents and Rh123 were substrates for efflux via a common MDR mechanism, they may compete for transport and thereby increase the Rh123 specific fluorescence compared with that of controls in the absence of azoles. Fluconazole, ketoconazole, and the experimental triazole ICI 153,066 were therefore added individually to *C. albicans* cells in PBS in the presence of Rh123, and the specific fluorescence was determined. In these experiments, the cells were subject only to short-term azole exposure, so the effects are not likely to be due to induced changes in the ergosterol content of the cell membrane.

Addition of azoles did not have a significant effect on Rh123 accumulation by *C. albicans* at a concentration of 10 μg/ml in either azole-resistant or azole-susceptible strains. However, when the azole concentration was increased 10-fold to 100 μg/ml, Rh123 accumulation increased significantly in the azole-resistant *C. albicans* strain 3302, from a specific fluorescence of 47 ± 3 (mean ± SE; n = 30) to 100 ± 14, 184 ± 12, and 120 ± 11 (n = 24 in each case) upon addition of fluconazole, ketoconazole, and ICI 153,066, respectively. Thus, azoles did influence Rh123 accumulation and may share an efflux transporter. However, the competition effect was evident only at high external concentrations of azole, which may have direct effects on the cell membrane. Therefore, we also attempted to show that Rh123 could influence the accumulation of fluconazole by the cells. [^{3}H]fluconazole accumulation was shown to have reached equilibrium after 80 min (data not shown), and the cells were therefore analyzed after 90 min. Uptake of [^{3}H]fluconazole by *C. albicans* 3153 and 3302 was measured in the presence and absence of Rh123 (130 μM). The presence of Rh123 had no effect on fluconazole accumulation in either strain (data not shown). Although intracellular concentrations of Rh123 and fluconazole were not determined, we conclude that Rh123 and fluconazole are unlikely to have a common efflux transporter in *C. albicans* 3302. The experiment was repeated using *C. glabrata* Y33.90 and Y33.91 (Fig. 3). The presence of Rh123 increased the accumulation of fluconazole in both the azole-susceptible (44% increase) and the azole-resistant (124% increase) strains, suggesting that in these organisms, fluconazole and Rh123 may compete for a common efflux mechanism.

Anticancer drugs that are known to be transported by mammalian P-gp (colchicine and daunomycin) and drugs to which *C. albicans* has been reported as being naturally resistant (cycloheximide and methotrexate) were also studied for their ability to influence Rh123 accumulation in the resistant *C. albicans* strain 3302. Cycloheximide and colchicine increased Rh123 accumulation in this strain (Fig. 4), suggesting that these drugs may compete with Rh123 for efflux and may therefore be cosubstrates for the efflux mechanism. However, daunomycin and methotrexate had no marked effect on Rh123 specific fluorescence (Fig. 4). None of these compounds had an effect on Rh123 accumulation in the azole-susceptible strain 3153 (data not shown).

Effects of respiratory inhibitors on Rh123 accumulation in *C. albicans*. The MDR phenotype is characterized by efflux that is coupled to ATP hydrolysis or membrane potential in different drug efflux systems (25, 34). To determine whether Rh123

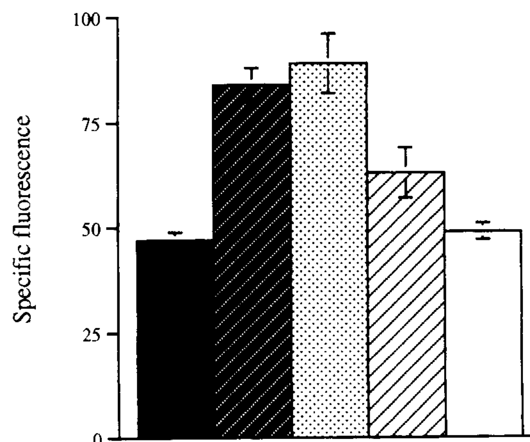

FIG. 4. Specific Rh123 fluorescence of yeast cells of *C. albicans* 3302 when grown in the presence of compounds that are known substrates of MDR transporters. Error bars show SE (n = 12) from one of three replicate experiments. ■, control; ▨, 30 μM cyclohexamide; ▧, 25 μM colchicine; ▨, 22 μM methotrexate; □, 18 μM daunomycin.

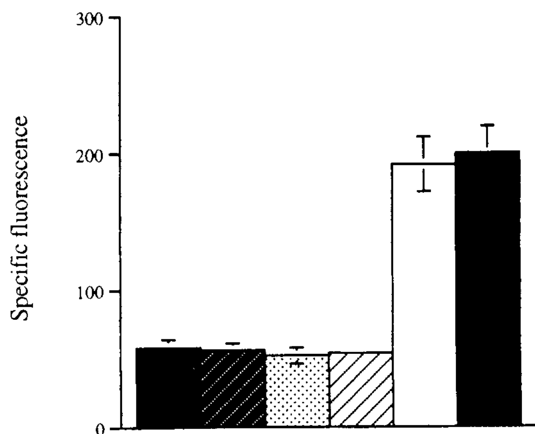

FIG. 5. Specific Rh123 fluorescence of yeast cells of *C. albicans* 3302 after exposure to inhibitors of oxidative phosphorylation and to proton uncouplers. Error bars show SE (n = 12) from one of three replicate experiments. Errors of <3% are not shown. From left to right, results are shown for the control, 0.5 μg of antimycin A per ml, 0.5 μg of potassium cyanide per ml, 1 mM sodium azide, 0.1 mM CCCP, and 0.1 mM FCCP.

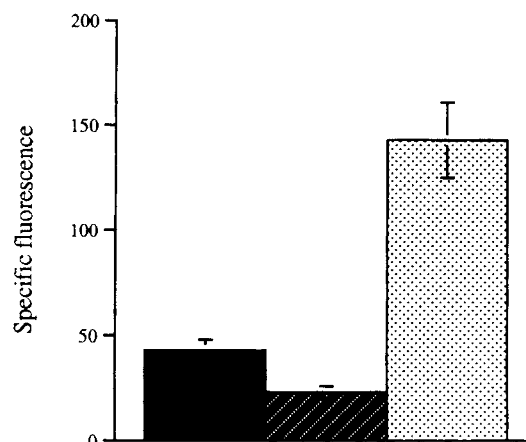

FIG. 6. Effects of known modulators of mammalian P-gp activity on the specific Rh123 fluorescence of mid-log-phase cells of *C. albicans* 3302. Error bars show SE (n = 12) from one of three replicate experiments. ■, control; ▨, 10 μM verapamil; ▤, 50 μM reserpine.

efflux in the resistant *C. albicans* strain 3302 was energy dependent, the effects of a variety of inhibitors on Rh123 accumulation were investigated. All inhibitors used were first tested for their effect on respiration of *C. albicans* yeast cells with an oxygen electrode, and ATP pools were measured after exposure to the inhibitors.

Rh123 accumulation in *C. albicans* 3302 was increased by the uncouplers FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone) and CCCP, but the electron transport chain inhibitors azide, cyanide, and antimycin A had little effect on Rh123 accumulation (Fig. 5). None of the inhibitors affected Rh123 accumulation in the sensitive strain 3153 (data not shown). The ATP pools were not significantly reduced 30 min after CCCP or FCCP addition, perhaps because of the fermentative growth of the cells under the conditions of the assay (data not shown). However, the data suggest that Rh123 efflux is dependent on the proton gradient across the cell membrane.

Effect of P-gp modulators on Rh123 accumulation by *C. albicans*. A variety of compounds, including the calcium channel blocker verapamil and the alkaloid reserpine, have been reported as having an inhibitory effect on P-gp and the MDR phenotype (10, 18, 21). The effects of these compounds on Rh123 accumulation by *C. albicans* were therefore examined (Fig. 6). Reserpine increased Rh123 accumulation significantly in the azole-resistant cells, but verapamil had no effect. Neither drug altered Rh123 accumulation in the susceptible strain (data not shown).

Rh123 accumulation in *C. krusei*. Accumulation of Rh123 in an azole-resistant and an azole-susceptible strain of *C. krusei* was examined. The azole-resistant strain, KM3, which had been isolated by selection on fluconazole-containing agar, accumulated significantly less Rh123 than strain CK5, the azole-susceptible parent (Fig. 7).

DISCUSSION

The incidence of azole resistance in pathogenic fungi has increased in recent years (19, 38). This reflects mainly the increased use of azoles to treat oropharyngeal and esophageal candidiasis in late-stage AIDS patients. The mechanisms of azole resistance in a number of organisms, including *C. albicans* and *C. glabrata*, have been studied (51). Changes in the target enzyme, P-450_{DM}, may decrease the binding affinity of azoles (22, 50). Alternatively, mutations causing overexpression of P-450_{DM} may occur, although they have been shown to only partially account for the azole resistance phenotype (51). Resistance may also occur by changes in the sterol synthesis pathway which compensate for the lack of ergosterol. For example, mutations in the $\Delta^{5,6}$ sterol desaturase lead to accumulation of fecosterol, which can partially counteract the effect of reductions in ergosterol content, as can 14-methylfecosterol, in azole-treated cells (22, 27). Finally, a number of strains have been shown to have reduced cytoplasmic concentrations of azoles (22, 45, 50). In one such *C. glabrata* strain, fluconazole resistance has been shown to result from an MDR-like energy-dependent efflux of the drug (14). In this study, we have investigated further the possibility that low intracellular concentrations of azoles may, in some cases, be mediated by the activity of a P-gp pump capable of effluxing azole antifungal compounds.

An assay was developed utilizing the fluorescent dye Rh123, which is known to be transported by a number of MDR organisms, from archaebacteria (33) to mammals (28). De-

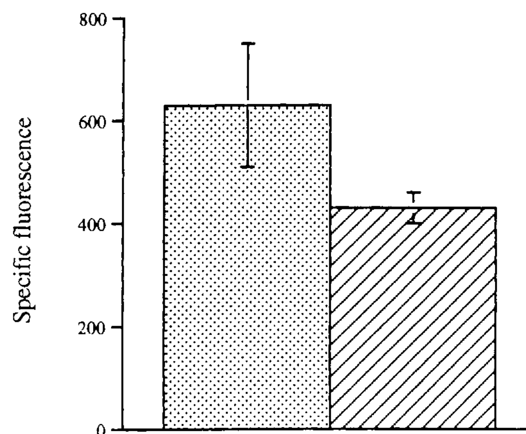

FIG. 7. Specific Rh123 fluorescence of mid-log-phase yeast cells of *C. krusei*. Error bars show SE (n = 12) from one of at least three replicate experiments. ▤, strain CK5 (azole susceptible); ▨, strain KM3 (azole resistant).

creased Rh123 accumulation has been used to diagnose MDR cell types (12, 16, 28, 33, 36), and reciprocal correlations between Rh123 accumulation and expression of P-gp in human tumor cells have been reported (16).

We showed that azole-resistant strains of *C. albicans*, *C. glabrata*, and *C. krusei* accumulated less Rh123 than equivalent azole-susceptible ones. In resistant *C. albicans* 3302, Rh123 accumulation was increased by proton uncouplers, and an MDR transporter may account for the lower cytoplasmic concentrations of Rh123 in this organism. Respiratory inhibitors sodium azide and antimycin A have been reported to inhibit P-gp activity of mammalian P-gp (1, 47). However, not all efflux proteins are energized directly by ATP; for example, the MDR phenotype in *B. subtilis* is encoded by a pump with sequence similarity to the tetracycline efflux pump and is energized by membrane potential and proton motive force (34). During batch culture, yeast cells of *C. albicans* undergo a marked decline in respiratory activity after the earliest stage of logarithmic growth (3, 54). This suggests that under these conditions, any Rh123 efflux may not be energized directly by ATP but may depend on the proton gradient across the cell membrane and be carried out by a protein of the MFS type.

A wide variety of compounds are reported as modulators of MDR transporter activity (18), including verapamil (9) and reserpine (40). In our experiments, Rh123 accumulation in *C. albicans* was not affected by exposure to verapamil. However, the level of verapamil permeability in *C. albicans* is very low (14a), and cytoplasmic concentrations sufficient to affect MDR may require prolonged exposure at high external concentrations. Reserpine increased Rh123 accumulation in the resistant *C. albicans* strain to levels approximating that in the azole-susceptible strain. In addition, interpretation of these observations is difficult, since the pharmacological effects of these drugs on fungi are not known.

Short-term exposure of *C. albicans* yeast cells to fluconazole, ketoconazole, and itraconazole affected specific Rh123 fluorescence but only at levels of drugs well above therapeutic concentrations. However, in a subsequent experiment, Rh123 had no effect on the accumulation of fluconazole by *C. albicans*, implying that the two compounds do not share an efflux mechanism. Rh123 accumulation in *C. albicans* was increased significantly by colchicine, a known substrate of mammalian P-gp (25) and cycloheximide, against which *C. albicans* has a naturally high resistance (11). These observations are consistent with the presence of an MDR transporter of the MFS type in *C. albicans* 3302 that utilizes a proton gradient to energize the efflux of Rh123, colchicine, and cyclohexamide from the cells. This transporter does not, however, appear to be involved in azole resistance in the strain. At this stage, we cannot rule out the existence of a separate transport protein in *C. albicans* that causes efflux of azoles from the cells. Alternatively, it is possible that the differential permeability of *C. albicans* 3153 and 3302 to azoles may be related to other factors, such as differences in the membrane lipid composition (23).

An azole-resistant strain (Y33.91) of *C. glabrata* was also shown to accumulate significantly less Rh123 than an azole-susceptible strain (Y33.90). In contrast to the results obtained with *C. albicans*, Rh123 and fluconazole showed competition for efflux from the azole-resistant *C. glabrata* strain. This organism has been shown to possess an MDR-like activity that results in efflux of fluconazole and benomyl from the cells (14). The results presented in this paper suggest that the same efflux mechanism also transports Rh123.

In summary, our results show that in some *Candida* strains, azole resistance is correlated inversely with the accumulation of Rh123, a known substrate for MDR proteins in a phylogenetically diverse group of organisms, from humans to archaebacteria. In azole-resistant strains of *C. albicans* and *C. glabrata*, there is active efflux of Rh123, consistent with the action of an MDR transporter. Furthermore, this efflux appears to play a role in azole resistance in *C. glabrata* but not in *C. albicans*. It is possible, however, that azole resistance in *C. albicans* is mediated by a separate efflux protein, since several different MDR transporters have been found in *S. cerevisiae* (5). The identification and analysis of genes encoding such efflux pumps will help to test this hypothesis.

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