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Cross-Resistance to Polyene and Azole Drugs in *Cryptococcus neoformans*

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Fluconazole was observed to inhibit sterol 14α-demethylase in the human pathogen *Cryptococcus neoformans*, and accumulation of a ketosteroid product was associated with growth arrest. A novel mechanism(s) of azole and amphotericin B cross-resistance was identified, unrelated to changes in sterol biosynthesis, as previously identified in *Saccharomyces cerevisiae*. Reduced cellular content of drug could account for the resistance phenotype, indicating the possible involvement of a mechanism similar to multidrug resistance observed in higher eukaryotes.

Infection with *Cryptococcus neoformans* leading to cryptococcal meningitis has been associated with up to 8% of all AIDS cases (7). Treatment failures and recurrence of infection in AIDS patients on current chemotherapeutic programs involving the use of azole antifungal agents or the polyene antibiotic amphotericin B are increasing (1, 3, 12, 21).

Ergosterol is the principal sterol in most fungi (for a review, see reference 9). Amphotericin B has been shown to bind ergosterol in the membrane, with resistance occurring through mutation in the biosynthetic pathway (3). Mode-of-action studies have shown that azole antifungal agents bind to cytochrome P-450 (P-$450_{14\alpha\text{-dm}}$), preventing sterol 14α-demethylation (20). Subsequent 4-demethylation may still occur, with formation of abnormal 14α-methyl sterols such as obtusifoliol, 14α-methylfecosterol, and the presumed product of attempted $\Delta^{5(6)}$ desaturation of 14α-methylfecosterol, 14α-methyl-3,6-diol (17). The accumulation of this last sterol has been shown genetically and biochemically to be associated with the arrest of growth in azole-treated *Saccharomyces cerevisiae* (17).

Lesions in P-$450_{14\alpha\text{-dm}}$ confer resistance to polyene antibiotics by preventing the formation of ergosterol, which is the target molecule for polyene action. For viability, these strains require a second defect in sterol $\Delta^{5(6)}$-desaturase which prevents the formation of 14α-methyl-3,6-diol (14). Azole-resistant mutants generated directly in *S. cerevisiae* are also defective in sterol $\Delta^{5(6)}$-desaturase (18); these mutants do not accumulate ergosterol and are also cross-resistant to polyene antifungal agents.

In the study described in this report we examined the potential for cross-resistance between azole and polyene antifungal agents in the fungal pathogen *C. neoformans*. A different pattern of sterol accumulation was observed following azole treatment of *C. neoformans* compared with that observed in *S. cerevisiae*, suggesting that the mechanisms for avoiding the formation of 14α-methyl-3,6-diol were not applicable (17). However, mutants which were cross-resistant to the azoles and amphotericin B were isolated, indicating an important consideration for future antifungal therapy.

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MATERIALS AND METHODS

Culture conditions. Two strains of the human pathogen *C. neoformans* (B4476 and B4500) were obtained from K. J. Kwon-Chung, National Institutes of Health, Bethesda, Md. Growth was supported on 2% (wt/vol) glucose, 2% (wt/vol) Difco peptone, and 1% (wt/vol) Difco yeast extract as liquid medium (YEPD medium) at 37°C. Cultures were shaken at 150 rpm. MICs were determined for cells harvested in the late log phase by centrifugation at 4,000 rpm and inoculation into 2 ml of YEPD medium to give a final concentration of 10^5 cells ml^{-1}. The azole antifungal agents used in the study were fluconazole (Pfizer, Sandwich, United Kingdom), itraconazole (Janssen Pharmaceutica, Wantage, United Kingdom), and ketoconazole (Janssen Pharmaceutica). Stock azole solutions were prepared in the solvent dimethyl sulfoxide (DMSO) at concentrations of 10^{-3} M. The polyene antibiotic amphotericin B (Sigma, Poole, United Kingdom) was dissolved in DMSO to a stock concentration of 1.08×10^{-3} M. Triplicate treatments with azole doses of between 1×10^{-4} and 5×10^{-8} M and amphotericin B doses of between 1.08×10^{-8} and 5.4×10^{-5} M were used. Test cultures used for MIC determinations were incubated for 24 and 72 h at 37°C and were shaken at 150 rpm, with growth assessed by cell counts (hemocytometer) and by determining the number of CFU per milliliter.

Experiments to determine the effect of treatment with time when using a dose of fluconazole equal to the MIC were conducted under conditions identical to those described above, except that 1-liter cultures in 2-liter flasks were inoculated with 5×10^6 cells ml^{-1} and 100 ml of culture was harvested for sterol analysis and for dry cell weight and cell count determinations.

Mutant isolation. A total of 10^6 cells of strain B4476 were inoculated onto YEPD medium–2% (wt/vol) agar containing 0.55×10^{-5}, 1.1×10^{-5}, 1.65×10^{-5}, 2.2×10^{-5}, and 3.2×10^{-5} M amphotericin B. A total of 10^6 cells of strain B4500 were inoculated onto 20 ml of YEPD medium–2% (wt/vol) agar containing fluconazole at concentrations 5- and 10-fold greater than the MIC for strain B4500 (10^{-4} M). The plates were incubated at 37°C for 7 days.

Sterol isolation and analyses. The growth of each strain both in the presence and in the absence of fluconazole at the MIC was achieved by incubation at 37°C and shaking at 150 rpm for 10 h. Extraction of nonsaponifable sterols was done by the method outlined by Woods (19), with cells saponified at 90°C for 1 h with 3 ml of methanol–2 ml of 60% KOH–2 ml of 0.5% pyrogallol in methanol. Extraction was completed with three hexane (5 ml) extractions, and the product was evaporated to dryness under N_2.

Following silylation of samples with BSTFA (20 μl) in toluene (100 μl) for 1 h at 60°C, the sterols were analyzed by gas chromatography by using on-column injection onto a SGE OV1-BP capillary column (25 m by 0.32 mm) at 80°C. Following sample injection (1 μl), the oven temperature was increased from 80 to 260°C at 40°C min^{-1} before being programmed to increase at 3°C min^{-1} to 290°C. The carrier gas was hydrogen and was used at an inlet pressure of 0.3 kg cm^{-2}. Peaks were determined by flame ionization detection and were quantified with a VG Multichrom data system.

For gas chromatography-mass spectrometry, derivatized samples (0.5 μl) were injected with a split/splitless injector (270°C) onto an ALLTECH SE-52 bonded-phase fused silica capillary column (25 m by 0.32 mm). The carrier gas was helium (inlet pressure, 0.4 kg cm^{-2}), and the temperature program was 180 to 280°C at 5°C min^{-1}. Electron ionization mass spectra were obtained at 70 eV.

Peak analysis and identification were realized by referring to the current literature (4, 5, 8) for relative retention times and fragmentation ion patterns. Representative data from triplicate analyses are provided.

TABLE 1. Alteration in sterol profile with time under fluconazole treatment at the MIC for *C. neoformans* B4500[a]

Sterol type	Relative % sterol composition at times (h) of:						
	0	0.5	2	4	6	8	10
Ergostatetraenol	0.8	5.6	1.1	0.9	2.7	1.4	0.3
Ergosterol	81.0	73.1	76.3	56.4	36.7	24.5	14.9
Ergosta-8-enol		2.3			0.6	0.9	
Obtusifolione		Trace	0.3	3.3	15.7	17.3	34.4
Ergosta-7-enol	15.2	11.0	6.7	5.1	4.0	7.1	4.3
Obtusifoliol				2.4			
Eburicol	3.0	2.3	9.6	27.6	32.3	42.8	45.7
Unknown sterols	0.0	5.7	6.0	4.3	8.0	6.0	0.4

[a] Dry cell weights were 192 ± 2, 290 ± 2, 761 ± 4, 830 ± 7, 1,301 ± 2, 1,501 ± 14, and 1,514 ± 5 mg/100 ml at 0, 0.5, 2, 4, 6, 8, and 10 h, respectively. Cell counts (10^6) were 4.9 ± 0.1, 5.0 ± 0.1, 10 ± 0.2, 12 ± 0.1, 14 ± 0.1, 15 ± 0.1, and 15 ± 0.1 cells ml^{-1} at 0, 0.5, 2, 4, 6, 8, and 10 h, respectively. The fluconazole MIC was 10^{-5} M.

Ergosterol content of strains. A total of 10^6 cells ml^{-1} were inoculated into 100 ml of liquid YEPD medium in 250-ml conical flasks. These were incubated at 37°C and 150 rpm until growth reached the late log phase (5×10^7 cells ml^{-1}). Cells were harvested by centrifugation at 4,000 rpm and were resuspended in 3 ml of methanol. Sterol extraction was done by the method outlined by Woods (19), and the ergosterol content was estimated by UV scanning spectroscopy with reference to a standard curve for ergosterol.

Ergosterol content of media. Cells were inoculated in 50 ml of liquid YEPD medium in 25-ml flasks at 10^6 cells ml^{-1} and were incubated at 37°C with shaking at 150 rpm for 24 h. The medium was clarified by centrifugation at 7,000 rpm for 10 min, transferred to 1-liter round-bottom flasks, and freeze-dried (Maxi Dry freeze drier; FTS Systems). Extraction of sterols from the freeze-dried medium was done by the method outlined by Woods (19), with ergosterol determination conducted spectrophotometrically on a Philips PU8800 UV-visible spectrophotometer.

Cellular content of radiolabelled fluconazole. Cells were inoculated into 100 ml of YEPD medium in 250-ml flasks at 10^6 cells ml^{-1} and were incubated at 37°C with shaking at 150 rpm until the growth reached the stationary phase. Cells were harvested by centrifugation at 4,000 rpm, washed in phosphate buffer (pH 7.0; phosphate buffer tablets [pH 7.0]; BDH), and resuspended in 1 ml of phosphate buffer (pH 7.0) at a final concentration of 2×10^9 cells ml^{-1}. [^{14}C]fluconazole (a gift from Pfizer Central Research) was added to a concentration of 10^{-5} M from a 22.2-μCi/mg stock solution, and the mixture was incubated for 1 h at 37°C and shaken every 10 min.

Cells were harvested and washed in 5 ml of cold fluconazole (5×10^{-5} M) and were then collected on Whatman GF/C 1-cm filters by vacuum filtration. This washing step was repeated three times. Cells and filters were baked at 80°C for 5 min. Counts per minute were measured on a Beckman LS 1801 scintillation counter. Drug content in the cells was calculated as the number of picomoles of fluconazole per cell. Each strain was tested in triplicate.

RESULTS

The role of sterols in the process of growth arrest following azole treatment has been examined in detail in *S. cerevisiae* (17). Inhibition of P-450$_{14\alpha\text{-dm}}$ by azole resulted in the accumulation of 14α-methyl sterols, predominantly 14α-methyl-3,6-diol (8). *C. neoformans* growth inhibition by the azole drug fluconazole was examined to compare the response of this pathogen with that of *S. cerevisiae.*

Inhibition of sterol biosynthesis in *C. neoformans* by using the MIC of fluconazole. Treatment of *C. neoformans* with fluconazole at the MIC for strain B4500 resulted in a rapid accumulation of 14α-methyl sterols (Table 1). Growth arrest, as determined as the point at which both dry cell weight and cell number reached a static level, occurred by 10 h after fluconazole treatment. This growth prior to arrest during treatment presumably utilized residual ergosterol, and approximately two to three generations was observed, with a slowdown after 4 h. By 10 h, untreated cells had undergone four to five generations and continued to grow beyond that point, unlike cells under fluconazole treatment. The predominant products after 10 h of fluconazole treatment were eburicol (concentration, 45.7%) and the ketosteroid obtusifolione (concentration, 34.4%). This latter product has been observed previously following itraconazole treatment of *C. neoformans*, but its importance in the process of growth arrest was unclear (15). In the present study the proportion of ergosterol decreased with treatment to below 20% of the total sterols when growth was arrested.

TABLE 2. Susceptibilities of in vitro-generated *C. neoformans* mutants of B4476 and B4500 to azole antifungal agents and the polyene antifungal agent amphotericin B

Strain	MIC (μM)			
	Azoles			Polyene, amphotericin B
	Fluconazole	Ketoconazole	Itraconazole	
B4476	1	0.1	0.1	1.1
Mutant 2	5	0.5	0.5	22
Mutant 5	5	0.5	0.5	32
B4500	1	0.1	0.1	1.1
Mutant 7	10	1.0	1.0	22
Mutant 8	10	1.0	1.0	32

Investigation of amphotericin B and azole cross-resistance. In order to investigate the potential for cross-resistance to polyene antibiotics and azole antifungal agents in *C. neoformans*, a screen for these mutants was undertaken. Two cross-resistant mutants of B4476 were isolated (mutants 2 and 5). They exhibited resistance to polyenes growing at concentrations 20- and 30-fold, respectively, greater than the MIC for B4476. Two cross-resistant mutants of B4500 (mutants 7 and 8) were isolated initially on the basis of fluconazole resistance at an azole concentration 10-fold greater than the MIC for the parent strain. The cross-resistance of all the isolates to azole antifungal agents (itraconazole, fluconazole, and ketoconazole) and amphotericin B was observed (Table 2).

Biochemical investigation of the basis of resistance. The sterol profiles of each mutant were investigated in either the absence or the presence of the fluconazole MIC identified for the parent strain (Table 3). The mutant strains were observed to have parental sterol profiles and were not defective in the biosynthetic pathway. Under treatment they showed a reduced level of accumulation of 14α-methylated sterols compared with that of the parent strain. The 14α-methylsterol:desmethyl sterol ratios were calculated to be 6:4, 1:3, and 1:3 for the B4476 parent strain, mutant 2, and mutant 5, respectively, and 4:1, 1:3, and 3:17 for the B4500 parent strain, mutant 7, and mutant 8, respectively. The mutants retained higher relative percentage compositions of ergosterol under treatment than the parent strains (Table 3). Overall, this indicated weak inhibition of sterol 14α-demethylase in each mutant compared with that in the parent strains. Some other variations in minor sterols between the parent and mutant strains were observed, but no pattern related to the cause of resistance was apparent.

Recently, overproduction of sterols has led to an azole resistance phenotype in tobacco through a presumed gene dosage effect (10). To examine if such a mechanism occurred in these mutants, ergosterol levels were quantified. The ergosterol concentration remained similar in each case, with values of 3, 2, and 2 μg of ergosterol per mg of cell (dry weight) for B4476, mutant 2, and mutant 5, respectively, and 2, 3, and 3 μg of ergosterol per mg of cell (dry weight) for B4500, mutant 7, and mutant 8, respectively. The possible efflux of excess ergosterol into the medium could also provide a potential route for amphotericin B resistance because high levels of exogenous

TABLE 3. Sterol compositions of *C. neoformans* B4476, B4500, and their respective cross-resistant mutants both in the absence and in the presence of fluconazole at the MIC[a]

Sterol	Relative % sterol composition											
	B4476 wild type		Mutant 2		Mutant 5		B4500 wild type		Mutant 7		Mutant 8	
	No drug	With FCZ[b]	No drug	With FCZ	No drug	With FCZ	No drug	With FCZ	No drug	With FCZ[a]	No drug	With FCZ
Ergostatetraenol	3.4	2.7	1.9	2.9	1.2	2.5	0.8	0.3	2.0	2.3	1.4	7.7
Ergosterol	66.3	25.0	68.4	57.2	69.5	48.6	81.0	14.9	62.7	65.8	68.0	71.3
Ergosta-8-enol	10.3	6.8	2.7		2.0	1.8			2.5	0.9	1.9	
Obtusifolione		18.1						34.4		3.3		4.3
Ergosta-5,7-dienol			2.9	10.2	2.3	5.7			2.5		2.1	
Ergosta-7-enol	12.2	3.7[c]	8.8		6.7	12.4[c]	15.2	4.3	8.7	4.6[c]	6.4	1.5
M = 484			6.7		8.3				9.8		9.6	
Eburicol	1.0	37.3	3.7	24.6	4.9	24.2	3.0	45.7	6.4	20.3	5.6	9.8
Unidentified sterols	6.5	7.2	4.9	5.1	5.1	4.8	0.0	0.4	5.4	2.8	5.0	5.4

[a] MIC, 10^{-6} M.
[b] FCZ, fluconazole.
[c] Mixture of ergosta-7-enol and $[M^+] = 496$.

ergosterol might bind amphotericin B, and thereby decrease the concentration available for cell inhibition. However, analysis of the medium did not detect such an ergosterol efflux.

Resistance involving the decreased influx and/or increased efflux of azole has previously been associated with azole resistance in some mutants of *Penicillium italicum* (2) and, possibly, *Candida glabrata* (16). Investigation of the cellular content of fluconazole in the *C. neoformans* parent strains compared with that in each mutant showed a marked fourfold difference in the levels between mutants and parent strains B4476 and B4500. The accumulation levels were 12.8 ± 2, 3.1 ± 0.5, 3.5 ± 0.5, 11.3 ± 2, 3.3 ± 0.4, and 3.7 ± 0.7 pmol of fluconazole per 10^9 cells in strain B4476, mutant 2, mutant 5, strain B4500, mutant 7, and mutant 8, respectively.

DISCUSSION

Azole treatment of *S. cerevisiae* results in the accumulation of lanosterol, obtusifoliol, 14α-methylfecosterol, and 14α-methyl-3,6-diol. The absence of the last three sterols in fluconazole-treated *C. neoformans* indicated a block in this strain at the 4-demethylation step of 14α-methylated-sterols, unlike *S. cerevisiae*, in which this reaction is not inhibited and subsequent sterol $\Delta^{5(6)}$ desaturation can be attempted. This inhibition of 4-demethylase may be caused by the azole compound or, as with inhibition of *S. cerevisiae* sterol $\Delta^{5(6)}$-desaturase, by the retention of a 14α-methyl group in the sterol.

Overall, the process of growth arrest in strain B4500 appeared to result from ergosterol depletion combined with an accumulation of nonutilizable sterol (eburicol) and the ketosteroid obtusifolione. Both of these compounds are unable to replace ergosterol's bulk membrane functions because they are methylated at C-4, whereas optimal membrane function requires 4-demethylation (6). The C-3 ketone moiety of obtusifolione prevents normal incorporation into the membrane because of its inability to act as a hydrogen donor in hydrogen bonding to the ester carbonyl moiety of the fatty acid associated with the phospholipid (a role normally fulfilled by the C-3 βOH group of sterols). 3-Ketosteroids further prevent membrane function through direct destabilization of the lipid bilayer structure (15). The different responses of *C. neoformans* and *S. cerevisiae* to azole treatment indicated that mechanisms of resistance involving the avoidance of 14α-methyl-3,6-diol accumulation were unlikely to be relevant for this pathogen.

Resistance to azole fungicides or amphotericin B could theoretically occur through a variety of mechanisms which would not necessarily result in cross-resistance, for example, azole resistance through alterations in the target sterol 14α-demethylase with reduced affinity for azole antifungal agents or amphotericin B resistance caused by defects in other enzymes associated with sterol biosynthesis such as $\Delta^{8(7)}$-isomerase (3).

The ease of isolation of mutants of *C. neoformans* cross-resistant to azole and polyene antifungal agents has considerable clinical implications. Other studies have related amphotericin B resistance in clinical isolates to alterations of ergosterol biosynthesis (3), but this does not appear to be the only mechanism of resistance (11). Our study indicates yet another mechanism which also results in cross-resistance to the other major class of antifungal drug. The frequency with which cross-resistance occurred (10^{-8}) suggested that a single mutation was responsible for resistance to both the azoles and the polyene antifungal agents. The mechanism of azole resistance of the mutants could be explained by the decreased uptake and/or increased efflux of the azole rather than a lesion in the regulation of sterol biosynthesis. Studies on the cellular accumulation of amphotericin B were unfortunately precluded by the fact that a radiolabelled form of the compound was unavailable.

Further research is under way to investigate whether alterations in cell membrane proteins are detectable in the mutants, such as those associated with multidrug resistance phenomena (e.g., see reference 13). Comparative studies are also required to establish whether similar mechanisms of resistance are in evidence in other pathogens accumulating either ketosteroid or 14α-methyl-3,6-diol under azole treatment.

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