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Resistant P45051A1 activity in azole antifungal tolerant *Cryptococcus neoformans* from AIDS patients

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Abstract Azole antifungal compounds are important in the treatment of Cryptococcosis, a major cause of mortality in AIDS patients. The target of the azole drugs is P450 mediated sterol 14α-demethylase. We have investigated the P450 system of *Cryptococcus neoformans* with respect to azole tolerance observed in clinical isolates which were obtained following the failure of fluconazole therapy. The clinical failure was correlated with in vitro tolerance of azole antifungal when compared to wild-type strains. The microsomal P450 system was typical of yeast and fungi and fluconazole tolerance was not associated with defective sterol biosynthesis. The strains had slightly elevated P450 content and slightly reduced azole levels in the cells, but a clear cause for resistance was the increased level of drug needed to inhibit the sterol 14α-demethylase in vitro.

Key words: Cryptococcus; Sterol 14α-demethylase (P45051A1); Azole antifungal; Resistance

1. Introduction

Cryptococcal disease is the most common life-threatening fungal infection associated with AIDS [1]. *Cryptococcus neoformans* may gain access through inhalation, but with immunodeficiency disseminates widely, especially to the central nervous system causing meningitis. The main antifungal agents used to treat infection are amphotericin B, which has a mode of action involving binding to ergosterol [2], and azole antifungals, particularly fluconazole, which inhibit P450-mediated sterol 14α-demethylase [3]. Failure of clinical azole antifungal therapy has been increasing [4] and we have examined sterol biosynthesis and the P450 systems of various wild-type strains and of strains associated with therapeutic failure.

Resistance to azole antifungals has been relatively poorly understood at the molecular level except in *Saccharomyces cerevisiae*. Sterol 14α-demethylase is a member of the *CYP51A1* family of P450s [5] and *S. cerevisiae* mutants defective in this enzyme have been observed to be resistant to azole antifungals [6,7]. Azole resistance has been shown in mutants, selected directly, to be caused by defects in sterol $\Delta^{5,6}$ desaturase [8] and such mutations are required as suppressors in strains containing blocked sterol 14α-demethylase [9]. Complementation studies have shown that in the strains containing both sterol 14α-demethylase and sterol $\Delta^{5,6}$ desaturase that the latter defect is solely responsible for resistance [8]. The basis for the involvement of sterol $\Delta^{5,6}$ desaturase is it's role in the formation of 14α-methylergosta-8,24(28)-dien-3β,6α-diol which does not support growth. In sterol $\Delta^{5,6}$ desaturase mutants the formation of this abnormal sterol is blocked and 14α-methylfecosterol accumulates which supports growth.

Studies on other pathogenic fungi have indicated a variety of changes which maybe associated with resistance including altered cellular content of azole, altered P450 content or altered P450 (for review see [10]). In some cases more than one phenotypic change has been observed, even in parent and mutant comparison [11], but multiple mutation would appear unlikely as a common cause for clinical resistance on grounds of probability. As the association of P450 lesion with sterol desaturase mutation has been observed in *Candida albicans* [10] similarity to the situation in *S. cerevisiae* may occur. One disturbing prospect is the possibility of cross-resistance to azole antifungal and amphotericin B which could result from defective sterol $\Delta^{5,6}$ desaturation [8].

One potential difference between the mode of action of azole antifungals in *C. neoformans* and *S. cerevisiae* and other fungi accumulating the abnormal diol under treatment e.g. *C. albicans*, *C. glabrata* and *A. fumigatus* [10] also stimulated our interest. This was the observation of ketosteroid at high levels rather than the diol [12] suggesting alternative mechanisms of resistance may arise as the formation of ketosteroids during C4 demethylation proceeds the formation of substrate for $\Delta^{5,6}$ desaturation. We describe here our studies on characterisation of azole antifungal tolerance in *C. neoformans* from AIDS patients who failed azole antifungal therapy.

2. Materials and methods

2.1. Strains

Three azole sensitive strains were used; B4500 (Serotype D, 24), A18 (Serotype A, from AIDS patient) and N7 (Serotype A, from non-AIDS patient). Four clinical isolates (Serotype A) were used from AIDS patients who failed fluconazole therapy and subsequently succumbed to Cryptococcosis; R715, R716, R717 and R718. Strains were routinely cultured on YEPD medium comprising 2% (w/v) glucose, 2% (w/v) Difco peptone and 1% (w/v) Difco yeast extract.

2.2. Chemicals

All chemicals were purchased from Sigma Chemical Company unless otherwise stated. Ketoconazole and itraconazole were purchased from Janssen Pharmaceutica and fluconazole was a gift from Pfizer Central Research.

2.3. Cell inhibition studies

Stationary phase cells, obtained from plate cultures incubated at 37°C for 3 days were resuspended at 5×10^3cells/ml in YEPD medium. Treatment with various doses of antifungal compound occurred over 3 days in 2 ml of medium in a Sterilin 60 ml container. Incubation was at 37°C, 150rpm and growth assessed by cell counts and colony-form-

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ing units/ml. Each test was repeated at least three times and minimum inhibitory concentrations were constant.

2.4. Cellular content of fluconazole

The cellular levels of fluconazole were investigated using [^{14}C]fluconazole (specific activity 22.2 μCi/mg). 10^9 cells were incubated in triplicate with 2×10^{-5} M [^{14}C]fluconazole in 0.1 M potassium phosphate buffer pH7 at 37°C, 150 rpm. The cells were harvested by centrifugation and washed three times in 10 ml 10^{-4} M unlabelled fluconazole prior to collection on Whatman GFC filters. This procedure was used to establish a favourable fluconazole concentration gradient to reduce loss of radiolabelled fluconazole from the cells and to wash off non-specifically bound fluconazole. Cellular azole content, a balance of uptake and efflux, was monitored with time and had reached a plateau by 30 min. Use of autoclaved cells indicated a background of nonspecific binding at less than 10% of the values shown. The samples were assayed for radioactivity on a Philips 4700 Scintillation Counter and efficiency was estimated using the external standard method.

2.5. Cell-free extracts and microsomal fractions

C. neoformans strains were grown in YEPD medium in 1 liter cultures at 37°C, 150 rpm in 2 liter flasks. Cells were harvested when growth was at 10^8 cells /ml and washed in sterile water. Cell homogenisation of batches of 20 g wet wt. of cells was carried out using a Braun disintegrator (Braun GmbH, Mesungen, Germany) operating at 4000 rpm with 4 × 30 s bursts with liquid carbon dioxide cooling. 20 g of glass beads (0.45–0.5 mm) were mixed with the cells and made up to 50 ml using 20% glycerol, 100 mM phosphate buffer pH 7.4. Cell free extracts were obtained following centrifugation at $1500 \times g$ and protein concentration determined using bicinchoninic acid method (Sigma). For microsomal fractions the cell-free extract was subject to further centrifugation, first at $10000 \times g$ to pellet mitochondria and subsequently at $100000 \times g$ to produce the microsomal pellet. This was resuspended to approximately 10 mg protein/ml in 20% glycerol, 100 mM phosphate pH 7.4 and P450 estimated using the method of Omura and Sato [15].

2.6. Inhibition of 14α-demethylase activity

Azole antifungal inhibition of P450 was investigated by assessing the cell-free biosynthesis of ergosterol, according to methods similar to previous studies [11,16,17]. After centrifugation at $1500 \times g$ for 5 min the cell-free biosynthesis of ergosterol was assayed. The reaction mixture consisted of cell-free extract (924 μl/ml) and cofactor solution (50 μl;containing 1 μmol NADP; 1 μmol NADPH; 1μmol NAD, 3μmol glucose 6-phosphate, 5 μmol ATP and 3 μmol reduced glutathione in distilled water). The complete mixture was adjusted to pH 7 (by addition of 10 M KOH), divalent cation solution (10 μl of 0.5 M $MgCl_2$ and 5 μl of 0.4 M $MnCl_2$), solution of azole compound dissolved in dimethylsulphoxide (1 μl) and [2-C^{14}]mevalonate (10 μl; 0.25 μCi, 4.7 nmol). The mixture was incubated in a water bath at 37°C for 2 h with shaking (110 rpm), after which the reaction was stopped by adding 1ml of a freshly prepared saponification reagent (15% (w/v) KOH in 90% (v/v) ethanol). Non-saponifiable lipids (sterols and sterol precursors) were then extracted with 2 × 3 ml heptane and dried under nitrogen. The non-saponifiable lipid was applied to silica gel thin layer chromatography (TLC) plates (ART 573, Merck) and developed using cyclohexane: diethyl ether 9:1 (v/v). Radioactive metabolites were located by autoradiography and excised for scintillation counting. The production of 4-desmethyl sterol was assessed for inhibition as described previously [18] and comprised more than 30% of the sterol produced. Experiments were performed in triplicate and IC_{50} values for ergosterol biosynthesis calculated.

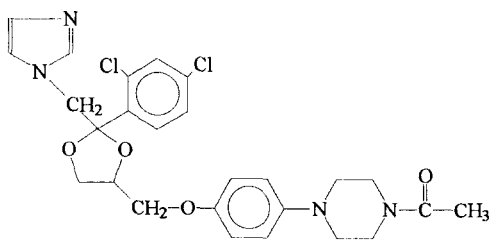
2.7. Identification of sterols by GC/MS

Samples for GC/MS were prepared from 100 ml cultures in the exponential phase of growth. The sample was saponified in 15% (w/v) KOH in 90% (v/v) ethanol at 80°C for 1h. Non-saponifiable lipids (sterol and sterol precursors) were then extracted with 2 × 5 ml heptane and dried under nitrogen. For determination of sterol composition a Hewlett/Packard GC/MS was used containing an Ultra 1 capillary column (10 m × 0.2 id) on a temperature programme 50°C (1 min) increased by 40°C/min to 290°C with a total run time of 17 min. Injection port temperature was 280°C (splitless) and the carrier gas was helium at 40 kPa.

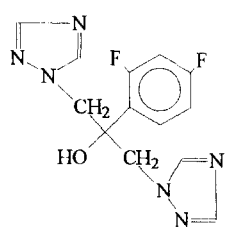
3. Results

3.1. In vitro tolerance of azoles

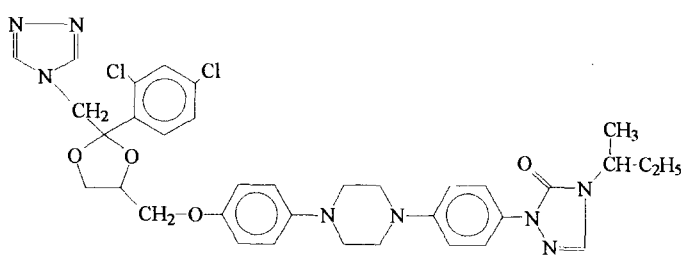
The *C. neoformans* wild-type and clinical isolates were examined for their azole MICs with the commonly used, orally



Ketoconazole



Fluconazole



Itraconazole

Fig. 1. Structural formulae of azole antifungal compounds used.

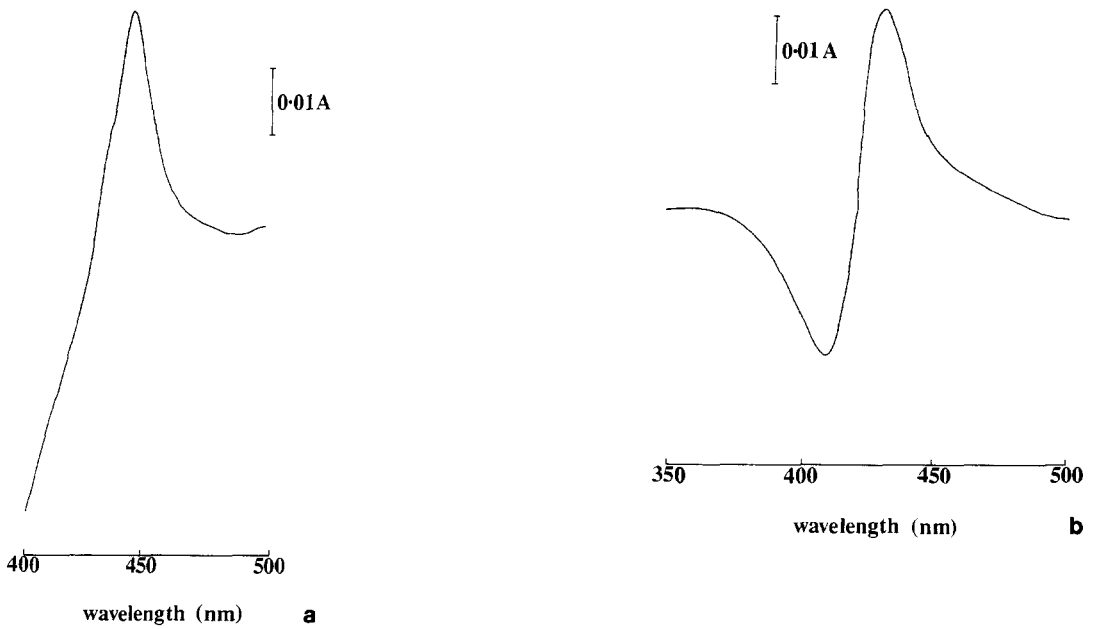


Fig. 2. (a) Carbon monoxide difference spectrum of reduced cytochrome P450 in microsomes prepared from the *C. neoformans* isolate R715. (b) Type II difference spectrum produced by the interaction of fluconazole with microsomes from the clinical isolate R717.

administered azole antifungals; fluconazole, itraconazole and ketoconazole (Fig. 1). In common with other studies fluconazole was the least potent inhibitor of growth of the three, but for all antifungals the clinical isolates R715, R716, R717 and R718 showed tolerance in comparison to N7, A18 or B4500. The MICs observed for the clinical isolates was approximately tenfold higher than for N7, A18 or B4500 (Table 1a). The biochemical basis of this tolerance was then investigated, but did not involve degradation of azole as indicated following incubation of [^{14}C]fluconazole with the strains (data not presented).

Table 1a
The minimum inhibitory concentration values of fluconazole, ketoconazole and itraconazole to growth of *C. neoformans* strains

	Minimum inhibitory concentration (μM)		
Strain	Fluconazole	Ketoconazole	Intraconazole
B4500	2.0	0.25	0.2
N7	1.0	0.1	0.1
A18	1.0	0.1	0.1
R715	20.0	2.5	5.0
R716	10.0	2.0	2.0
R717	20.0	2.0	5.0
R718	20.0	4.0	5.0

3.2. Sterol composition of the strains

Sterol analysis did not indicate any overall change in the azole-tolerant clinical isolates as might be conferred by stringent blocks or leaky mutations in enzymes of the ergosterol biosynthetic pathway (Table 1b). All strains accumulated approximately 70% ergosterol in accord with our previous analysis of wild-type *C. neoformans* [13].

3.3. Study of C. neoformans microsomal P450

Using mechanical breakage it was possible to produce microsomal fractions of the different isolates which displayed reduced carbon monoxide difference spectra with a Soret maximum at 448nm (Fig. 2a). Problems associated with cytochrome oxidase contamination which obscure this peak were observed if breakage conditions were too harsh. All the microsomal fractions exhibited typical Type II spectra when azole antifungal was added indicating binding to the haem as a sixth ligand (Fig. 2b; [10]). The specific content of P450 in the fractions was observed to be higher in the azole tolerant isolates, approximately twice the level of N7, A18 and B4500 (Table 2). The specific content observed in all the *C. neoformans* strains was comparable to the strains of *S. cerevisiae* which have the highest levels of microsomal P450 [14].

Table 1b
Sterol composition of *C. neoformans* strains

	Percentage sterol composition of wild type and azole-tolerant isolates						
	B4500	A18	N7	R715	R716	R717	R718
Ergosterol	70.0	64.0	79.5	71.5	75.6	76.6	71.4
Ergosta-7,22-dienol	3.0	–	–	–	–	–	–
Ergosta-5,7-dienol	2.0	–	–	–	–	–	–
Ergosta-7-enol	13.5	4.2	11.0	16.4	14.4	13.0	17.4
4-Methylergosta-8,24(28)-dienol	5.0	2.0	2.0	–	–	–	–
4,4-Dimethylergosta-8,24(28)-dienol	6.5	2.1	7.5	–	–	–	–
24-Methylene-24,25-dihydrolanosterol	5.0	27.7	–	–	–	–	–
Unknown	0.0	–	–	12.1	10.0	20.4	11.2

Table 2
Relationship of microsomal P450 cellular content and cellular [^{14}C]fluconazole levels for *C. neoformans* strains expressed as levels per cell

Strain	P450/Cell (pmol)	Fluconazole/Cell (pmol)
B4500	0.7×10^{-8}	2.4×10^{-7}
N7	0.7×10^{-8}	2.2×10^{-7}
A18	0.6×10^{-8}	2.1×10^{-7}
R715	1.4×10^{-8}	1.1×10^{-7}
R716	1.6×10^{-8}	1.2×10^{-7}
R717	1.5×10^{-8}	1.6×10^{-7}
R718	1.7×10^{-8}	1.4×10^{-7}

Table 3
IC_{50} values of fluconazole, ketoconazole and itraconazole on incorporation of [2-^{14}C]mevalonate into C4-desmethyl sterols in cell-free extracts of sensitive and tolerant *C. neoformans* strains

	IC_{50} values for in vitro ergosterol biosynthesis (nM)[a]		
Strain	Fluconazole	Ketoconazole	Itraconazole
B4500	400 ± 150	4.0 ± 2.0	3.7 ± 1.5
N7	300 ± 210	3.4 ± 2.3	2.3 ± 1.5
A18	600 ± 200	3.0 ± 1.7	4.1 ± 2.0
R715	5000 ± 3000	27.0 ± 12.0	27.0 ± 21.0
R716	3500 ± 2000	33.0 ± 21.0	31.0 ± 18.0
R717	3300 ± 2000	53.0 ± 11.0	43.0 ± 21.0
R718	5000 ± 3200	57.0 ± 20.0	47.0 ± 12.0

[a] Values shown ± standard deviation.

3.4. Comparison of azole content in cells

Cellular content of azole antifungal in the strains was investigated using [^{14}C]fluconazole. The time course of accumulation of fluconazole was investigated and observed to plateau after treatment for 30 min. The content of fluconazole was reduced in all the tolerant isolates in comparison to N7, A18 and B4500 (Table 2). However, the content of intracellular azole was still in excess of the microsomal P450 content when related to the amounts of each per cell (Table 2).

3.5. Investigation of inhibition of sterol 14α-demethylation

Using cell-free extracts of all the strains IC_{50} determinations for inhibition of ergosterol biosynthesis were made for itraconazole, ketoconazole and fluconazole. In all cases the IC_{50} values correlated with the observed in vitro tolerance of the strains and showed approximately tenfold differences in sensitivity between the tolerant strains R715, R716, R717, R718 and the wild-type strains N7, A18 and B4500 (Table 3).

4. Discussion

The present studies have demonstrated for the first time an alteration of P450 to be related to azole tolerance. The molecular basis of azole resistance in fungi is a subject of current interest due to its emergence as a clinical problem and also in agriculture [10]. Sterol $\Delta^{5,6}$ desaturase has been shown to play a critical role in azole mode of action and resistance in *S. cerevisiae*, being required for the formation of 14α-methylergosta-24(28)-dien-3β,6α-diol under azole antifungal treatment, which is incompatible with sterol function in membranes [15]. Mutants blocked in this enzyme do not synthesise the diol and accumulate the precursor sterol, 14α-methylfecosterol, which is capable of supporting growth. Previously, it has been shown that *C. neoformans* does not accumulate 14α-methyl-3,6-diol or 14α-methylfecosterol to a high level under azole treatment and thus other mechanisms of resistance must operate [12]. Ketosteroid accumulation, particularly for obtusifolione, to high levels and ergosterol reduction in azole-treated *C. neoformans* could be resisted by either azole degradation, a mutation altering sterol biosynthesis (apart from sterol 14α-demethylase), elevation of the cellular content of the target enzyme, alteration of affinity of the target enzyme for the drug or the development of mechanisms for reducing cellular content of the drug.

Tolerance was observed to be unrelated to azole degradation or to defective sterol biosynthesis in genes other than encoding sterol 14α-demethylase as was observed in *S. cerevisiae*. Our observation of ergosterol as the predominant sterol of all the strains studied is in accord with a previous study [12,13]. Others have observed lower levels of ergosterol [18], but this may have been due to harvesting cells in stationary phase where we have observed a similar effect. The accumulation of obtusifolione following azole treatment in *C. neoformans* indicates interference with the process of C4 demethylation where the last step involves a ketosteroid reductase [19]. Interference with this step may be due to a direct effect of azole, or more likely, inhibition caused by retention of the 14α-methyl group in the substrate as appears to occur for *S. cerevisiae* during attempted $\Delta^{5,6}$ desaturation [8]. Whatever the origin of the effect there appears to be a difference in sensitivity between the ketosteroid reductases of different fungal species. Previous studies have indicated that the 14α-methyl sterols preceeding this step, including eburicol, will not support growth [20] and this was consistent with our observations that a block to sterol biosynthesis was not associated with azole tolerance.

Gene dosage effects can result in increased resistance to azole antifungals, but elevation of the enzyme by 100-fold in a *S. cerevisiae* heterologous expression systems only altered the MIC fivefold [10]. The observation of a raised specific content of P450 in microsomal fractions from R715, R716, R717 and R718 provides some support for a similar role. However, the elevation would be unlikely to account for their resistance unless a dramatic increase in sterol 14α-demethylase was masked by the presence of other major P450 enzymes in the microsomal fractions of the wild-type strains. In *S. cerevisiae* sterol 14α-demethylase is the major P450 of vegetatively growing yeast [21], but confirmation that this also the case in *C. neoformans* will require further purification studies. Antibodies raised against the *S. cerevisiae* sterol 14α-demethylase do not cross-react with microsomal fractions of *C. neoformans* to allow any immediate immunological studies (unpublished observation).

Studies on *Candida albicans* [22] and *Penicillium italicum* [23] have observed reduced cellular levels of azole antifungal compounds associated with resistance. Such observations have led to the suggestion that alterations similar to those resulting in multiple-drug resistance are occurring [8]. Slightly reduced azole content per cell was observed for the azole tolerant strains, but no cross-resistance to other antifungal compounds like amphotericin B or cycloheximide (data not presented). Although this reduced accumulation of drug might be associated with the azole-tolerant phenotype, we examined the basis of this hypothesis one stage further by relating the azole content of the cells to their microsomal P450 content. Even when azole content was reduced it was still an order of magnitude in excess

of the P450 suggesting ample drug was available for growth inhibition. Without developing further theories related to the compartmentalisation of azole antifungal within the cell we considered the basis of tolerance was probably not related to the changes in cellular content of azole antifungal.

The in vitro inhibition of ergosterol biosynthesis provided an obvious cause of azole tolerance based on a change in concentration of azole required to inhibit sterol 14α-demethylation. R715, R716, R717 and R718 required approximately tenfold higher concentrations of azole in order to provide equivalent inhibition to N7, A18 and B4500 as was observed for variation in sensitivity in whole cells. This alteration may be due to changes in the level of the target P450 for azole antifungals, although alterations in the active site which retain catalytic activity, but have altered affinity for azole is perhaps more likely given the considerations above. Further studies are warranted to understand the molecular biology of the altered sterol 14α-demethylation step as well as extension to determine the extent of clinical relevance.

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