

# Resistance to fluconazole and cross-resistance to amphotericin B in *Candida albicans* from AIDS patients caused by defective sterol $\Delta^{5,6}$ -desaturation

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**Abstract** Fluconazole resistance occurs in >10% of cases of candidosis during the late stages of AIDS. We show here in two clinical isolates that resistance was caused by defective sterol  $\Delta^{5,6}$ -desaturation. This altered the type of sterol accumulating under fluconazole treatment from 14 $\alpha$ -methylergosta-8,24(28)-dien-3 $\beta$ ,6 $\alpha$ -diol to 14 $\alpha$ -methylfecosterol which is capable of supporting growth. A consequence of this mechanism of azole resistance is that an absence of ergosterol causes cross-resistance to the other major antifungal agent available, amphotericin B. The results also show that growth arrest after fluconazole treatment of *C. albicans* in clinical conditions is caused by 14 $\alpha$ -methylergosta-8,24(28)-dien-3 $\beta$ ,6 $\alpha$ -diol accumulation.

**Key words:** Fluconazole; Candidosis; Cross-resistance; P450 inhibition

## 1. Introduction

Azole antifungals have proved to be central to therapy for the rapidly increasing numbers of patients infected with fungi. Their mode of action is through inhibition of a cytochrome P450 enzyme (CYP51) which undertakes 14 $\alpha$ -demethylation of lanosterol in *Saccharomyces cerevisiae*, but 24-methylenedihydrolanosterol in most other fungi including the major human pathogen *Candida albicans* (for review, see [1]). Plants and mammals also have an equivalent activity, but the antifungals are selective inhibitors of the fungal enzyme. The consequence of inhibition is a reduction in the intracellular levels of ergosterol, the main sterol of fungi, and an increase in substrate and other abnormal sterols produced by further metabolism of the substrate, but without removal of the 14 $\alpha$ -methyl group. This change in sterol composition results in growth arrest.

Resistance to azole antifungals (Fig. 1A) has been an important problem in agriculture among phytopathogens, but for fluconazole it has also become a considerable obstacle to successful therapy in late-stage AIDS patients as more than 10% now develop mucosal candidosis resistant to fluconazole [2]. The best elucidated mechanism of azole resistance is from laboratory studies with *S. cerevisiae* where a change in sterol metabolism caused by mutation in sterol  $\Delta^{5,6}$ -desaturase causes resistance. The target enzyme was still inhibited, but

instead of accumulating 14 $\alpha$ -methylergosta-8,24(28)-dien-3 $\beta$ ,6 $\alpha$ -diol (14 $\alpha$ -methyl-3,6-diol), 14 $\alpha$ -methylfecosterol was produced which fulfilled the requirements of the cell for growth unlike the 6-hydroxylated sterol (Fig. 1B [3]). Sterol  $\Delta^{5,6}$ -desaturase mutations also act as suppressor mutations allowing viability of *S. cerevisiae* strains containing an otherwise lethal gene disruption in CYP51 which subsequently also synthesise 14 $\alpha$ -methylfecosterol [4]. Presumably, the retention of the 14 $\alpha$ -methyl group in the sterol interferes with attempted  $\Delta^{5,6}$ -desaturation in normal strains, resulting in 6-hydroxylation which is incompatible with sterol membrane function. Despite these *S. cerevisiae* studies, the general relevance of defective sterol  $\Delta^{5,6}$ -desaturase to azole antifungal resistance in practical settings has remained unclear.

Due to its therapeutic importance, a large effort is currently being made towards understanding the basis of resistance in *C. albicans* from the clinic. Reduced accumulation of drug has been observed in two clinical isolates [5] and altered levels of efflux pumps have been associated with resistance elsewhere, although without examining changes in sterol phenotype or target enzyme for azole drugs [6]. Considerable differences exist between laboratory cultures of *S. cerevisiae* and pathogenic *C. albicans* infecting a human host which might alter the types of mutants selected e.g. regarding sterol  $\Delta^{5,6}$ -desaturase. *C. albicans* is diploid, necessitating a dominant mechanism to operate and the conditions of growth differ considerably which might affect the chances for survival of certain strains. For example, *S. cerevisiae* mutants defective in sterol  $\Delta^{5,6}$ -desaturase are osmotically sensitive and are unable to grow on carbon sources requiring respiration, suggesting impaired mitochondrial function [7]. Despite these potential areas for difference we show here that this mechanism of resistance is present clinically in isolates from AIDS patients and can be expected at a high frequency.

## 2. Materials and methods

### 2.1. Strains

All *C. albicans* isolates were from AIDS patients. S1 and S2 were fluconazole-sensitive isolates of *C. albicans*, but R1 and R2 exhibited fluconazole resistance. R1 was from a 48 year old female, HIV positive, who had undergone fluconazole therapy at 100 mg/day rising to 400 mg/day over several years. The therapy failed as did amphotericin B therapy at 30 mg/day. R2 was from a 36 year old male, HIV positive, receiving repetitive fluconazole therapy for several years whose last treatment was 6 weeks at up to 800 mg/day. All cultures used RPMI-1640 medium (Sigma), unless specified.

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## 2.2. Chemicals

Unless specified all chemicals were obtained from Sigma Chemical Company, Poole, Dorset, UK.

## 2.3. Growth inhibition studies

Stationary phase cells obtained from plate cultures incubated at 37°C on RPMI-1640 medium with 2% (w/v) Difco Bactoagar were inoculated in 2 ml medium contained in 60 ml Sterilin containers at 5000 cells/ml. Treatment with various doses of antifungal compound occurred over 3 days at 37°C, 150 rpm and growth was assessed by cell counts and colony-forming units/ml on YEPD consisting of 2% (w/v) glucose, 2% (w/v) Difco Bactopeptone, 1% (w/v) Difco yeast extract and 2% (w/v) Difco Bactoagar. Each test was repeated at least three times and minimum inhibitory concentrations (MIC) were constant. Resistance of the strains was confirmed by E-test.

## 2.4. Identification of sterols by GC/MS

Samples for GC/MS were prepared from 50-ml cultures in the exponential phase of growth on RPMI-1650. Treated cells were exposed to fluconazole at 16 µg/ml over 24 h (in excess of the MIC for sensitive strains). The cell pellet was saponified in 15% (w/v) KOH in 90% (w/v) ethanol at 80°C for 1 h. Non-saponifiable lipids (sterols and sterol precursors) were extracted with 3×5 ml heptane and dried under nitrogen. Following silylation for 1 h at 60°C with BSTFA (50 µl) in 50 µl of toluene, sterols were analysed by GC/MS (VG 12-250 (VG Biotech) using split injections with a split ratio of 20:1. Sterol identification was by reference to relative retention time and mass spectra as reported previously [4,5].

## 3. Results

Table 1 shows the minimum inhibitory concentrations observed for the clinical isolates of *C. albicans* when investigated in vitro. They exhibited a range of sensitivities with isolates R1 and R2 being resistant to fluconazole on comparison to S1 and S2. Resistance to the other major antifungal drug amphotericin B was also observed, although the -fold increase in resistance was not as high as between sensitive and resistant strains in the fluconazole treatments.

Analysis of sterols present in the strains showed a clear difference between the sensitive strains S1 and S2 and the resistant strains R1 and R2 (Table 2). The pattern of sterols in S1 and S2 cells changed from predominantly ergosterol, when untreated, to containing a high concentration of 14 $\alpha$ -methyl-3,6-diol after treatment with fluconazole. R1 and R2 were clearly defective in sterol  $\Delta^{5,6}$ -desaturation accumulating mainly ergosta-7-enol and also ergosta-7,22-dienol when untreated with fluconazole. R2 also accumulated ergosta-7,24(28)-dienol, another sterol lacking  $\Delta^{5,6}$ -desaturation. Under treatment they did not accumulate the 14 $\alpha$ -methyl-3,6-diol, but instead formed the precursor 14 $\alpha$ -methylfecosterol which allowed them to continue growing in the presence of the drug. The substrate of sterol 14 $\alpha$ -demethylase, 24-methylenedihydrolanosterol (eburicol) and its C4-demethylated derivative, obtusifolione also accumulated.

Table 1

Minimum inhibitory concentrations of fluconazole and amphotericin B observed on treating the various clinical isolates of *Candida albicans* from AIDS patients

<i>C. albicans</i> isolate	Minimum inhibitory concentration (µg/ml)	
	Fluconazole	Amphotericin B
S1	4.0	0.5
S2	5.0	1.0
R1	62.0	4.0
R2	78.0	4.5

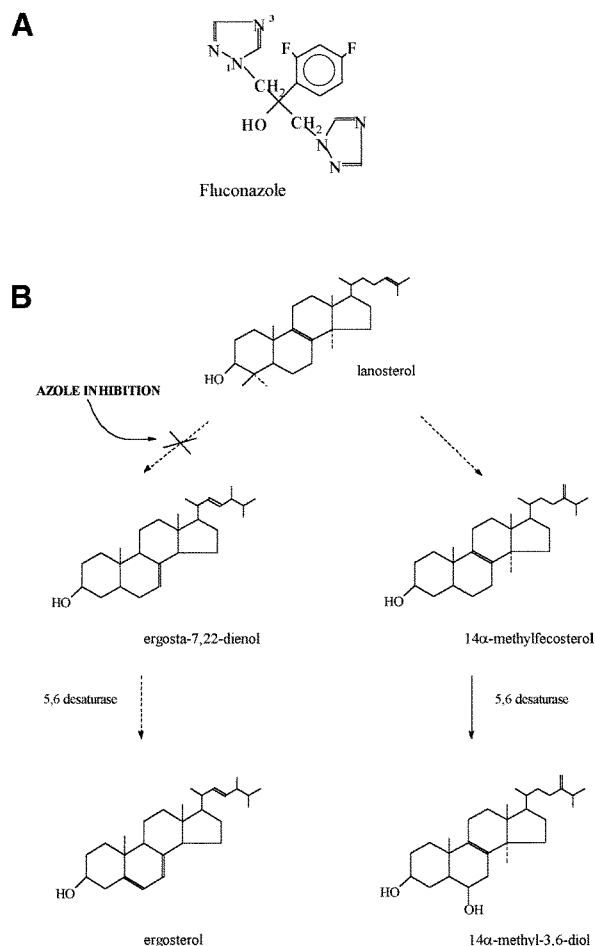


Fig. 1. (A) The structure of fluconazole and (B) the effect of fluconazole inhibition of ergosterol biosynthesis in *Saccharomyces cerevisiae*. Structures included are lanosterol, the substrate of sterol 14 $\alpha$ -demethylase in this yeast; ergosta-7,22-dienol, a substrate for sterol  $\Delta^{5,6}$ -desaturase; ergosterol, the end-product of the pathway and, from azole treated cells, 14 $\alpha$ -methylfecosterol and 14 $\alpha$ -methylergosta-8,24(28)-dien-3 $\beta$ ,6 $\alpha$ -diol.

## 4. Discussion

The findings here indicate that defective sterol  $\Delta^{5,6}$ -desaturation is a common mechanism of fluconazole resistance in *C. albicans* from AIDS patients, being observed in both resistant isolates analysed in this study. It also shows accumulation of 14 $\alpha$ -methyl-3,6-diol is responsible for growth arrest of this yeast following fluconazole treatment under clinical conditions as avoiding its accumulation allows continued growth. Previously, we observed a partial dominance effect for fluconazole resistance in a *S. cerevisiae* strain heterozygous for a sterol  $\Delta^{5,6}$ -desaturase mutation [3] and this may suggest a possible route towards the development of the phenotype observed here for the *C. albicans* strains R1 and R2. Initially, a resistance mutation may have been selected in one of the two alleles and subsequently gene conversion or further mutation involving the second allele could produce increased resistance in a daughter strain exposed over a prolonged period of treatment to fluconazole. Further analysis of the genes in the resistant strains will cast light on the origin of the defective alleles in these diploid pathogenic *C. albicans*. The absence of ergosterol in the resistant strains R1 and R2 can be attrib-

Table 2

Percentage composition of various sterols of the *C. albicans* clinical isolates with and without treatment with 16 µg/ml fluconazole

Sterol	<i>C. albicans</i> isolate							
	Without treatment				Following fluconazole treatment			
	S1	S2	R1	R2	S1	S2	R1	R2
Ergosterol	98.0	97.0			2.0	1.6		
Ergosta-7,24(28)-dienol				11.0				
Ergosta-7,22-dienol			77.0	74.0				
Ergosta-7-enol			22.0	12.0				
Euburicol					16.1	12.3	7.0	12.0
Obtusifolol					34.5	13.1	10.0	14.0
14 $\alpha$ -Methylfecosterol							82.0	71.0
14 $\alpha$ -Methyl-3,6-diol					45.2	72.0		
Unknown	2.0	3.0	1.0	3.0	2.2	1.0	1.0	3.0

uted as the cause of their cross-resistance to amphotericin B, as it is known that this polyene antibiotic acts through binding to ergosterol [8]. The medical implications of these observations are also discussed elsewhere [9].

The relative frequency of this mechanism of resistance among other potential mechanisms remains to be determined and in two other strains we analysed previously it did not operate; instead reduced intracellular accumulation of drug was observed and normal sterol biosynthesis [5]. The disturbing prospect of significant numbers of resistant strains being cross-resistant to amphotericin B as observed here is alarming and underlines the requirement for new antifungals with different modes of action. Additionally, there is a need for a survey of molecular mechanisms of resistance in order to determine the best therapeutic response.

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