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Defective sterol $\Delta^{5(6)}$desaturase as a cause of azole resistance in *Ustilago maydis*

Timothy Joseph-Horne [a], Nigel J. Manning [b], Derek Hollomon [c], Steven L. Kelly [a,*]

[a] *Krebs Institute for Biomolecular Research, Department of Molecular Biology and Biotechnology, University of Sheffield, Sheffield S10 2UH, UK*

[b] *Neonatal Screening Laboratory, Sheffield Childrens Hospital, Western Bank S10 2UH, UK*

[c] *Department of Agricultural Sciences, University of Bristol, Institute of Arable Crops Research, Long Ashton Research Station, Long Ashton, Bristol, UK*

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Abstract

Resistance to azole antifungals in *Ustilago maydis* was associated with a leaky defect in sterol $\Delta^{5(6)}$desaturase. This defect resulted in reduced accumulation of 14α-methylergosta-24(28)-diene-3β,6α-diol and an increase in the proportion of 14α-methylfecosterol in treated cells when compared to the parent strain. The results demonstrate the importance of this mechanism in pathogenic fungi.

Keywords: Azole; Resistance; P450; Sterol desaturase; *Ustilago maydis*

1. Introduction

The sterol biosynthetic pathway has provided a variety of target sites for the development of antifungal compounds. One major group of antifungal agents are the azoles and their derivatives, which inhibit a P450 enzyme, sterol 14α-demethylase (P450 $_{14\alpha\text{-dm}}$) [1]. Inhibition of this fungal enzyme results in reduced levels of ergosterol (the principle sterol of most fungi) and the accumulation of 14α-methyl sterols such as lanosterol, obtusifoliol and 14α-methyl-3,6-diol (Fig. 1) (for review, see [2]). The combination of sterols produced is unable to support growth [3].

Resistance to azole antifungals has become a significant problem in the treatment of patients suffering from AIDS, where fungal infection is common [4], as well as in agriculture [5]. The mechanisms of resistance remain unclear except in *Saccharomyces cerevisiae*, where resistance is conferred by mutation in sterol $\Delta^{5(6)}$desaturase which changes the type of 14α-methyl sterol accumulating under azole treatment [6]. Instead of accumulating 14α-methyl-3,6-diol, such mutants accumulate 14α-methylfecosterol as the formation of 14α-methyl-3,6-diol requires active $\Delta^{5(6)}$desaturase [7]. This change in sterol accumulating with treatment allows growth to continue utilising 14α-methylfecosterol. The ability of a le-

* Corresponding author. Tel.: +44 (742) 768 555; Fax: +44 (742) 728 697

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sion in $\Delta^{5(6)}$desaturase to confer resistance to azoles on inhibition of sterol 14α-demethylase is also reflected in the presence of sterol $\Delta^{5(6)}$desaturase mutations as suppressors allowing growth in mutant strains containing a gene disruption of sterol 14α-demethylase [8]. The resistance of such strains is not increased by the second defect in the $P450_{14\alpha\text{-dm}}$ [9].

Studies on azole resistance in other fungal pathogens have suggested roles for altered efflux [10] and cellular azole distribution [11]. Using polyene antibiotics, which act through binding to ergosterol [12], some mutants of fungal pathogens have also been produced with lesions in the pathway including the $P450_{14\alpha\text{-dm}}$; for example the azole-resistant mutant of *Ustilago maydis*, erg 40, which accumulates 14α-methylfecosterol [13]. However, as observed for sterol 14α-demethylase mutants of *S. cerevisiae* [9], this strain may also be defective in sterol $\Delta^{5(6)}$desaturase. Such double mutants have not been observed in any direct selection for azole resistance.

We report here results for *U. maydis* demonstrat-

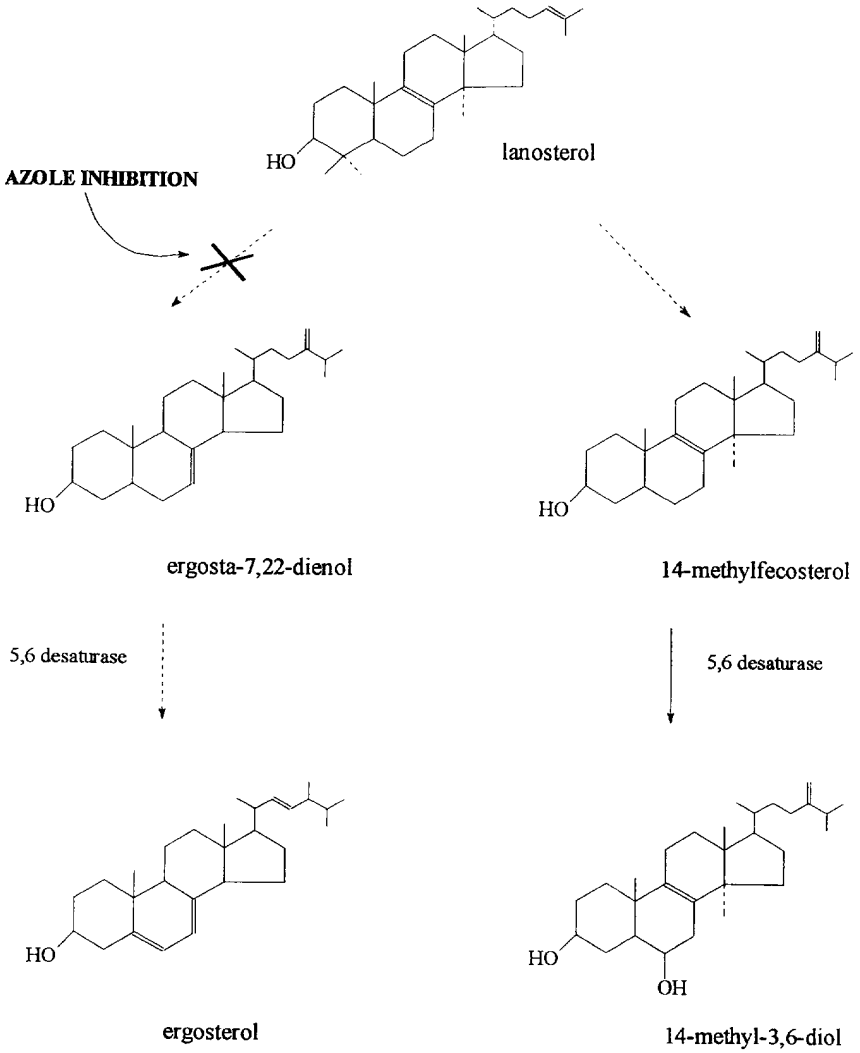

Fig. 1. Consequence of azole inhibition on sterol biosynthesis in *S. cerevisiae*.

ing the general relevance of the azole resistance mechanism established for *S. cerevisiae*. As other fungal pathogens such as *Candida albicans* and *Aspergillus fumigatus* accumulate 14α-methyl-3,6-diol under treatment (for review, see [2]), further examples can be anticipated. However, some significant differences in the inhibition of ergosterol biosynthesis were found which may result from differences in the biosynthetic enzymes in different fungal species.

2. Materials and methods

2.1. Strains

U. maydis strain ATCC 14826 was grown on liquid YEPD media (2% (w/v) Difco peptone, 2% (w/v) glucose and 1% (w/v) Difco yeast extract) at 25°C and 150 rpm.

2.2. Chemicals

Unless otherwise indicated, all azoles were supplied by their respective manufacturers as technical grades. Fenarimol, tebuconazole and prochloraz (in methanol), triadimenol and diclobutrazol (in dimethyl sulfoxide (DMSO)) were prepared as 10^{-4} M stocks. Amphotericin B was prepared as a 1 mg ml^{-1} stock in DMSO. All reagents were obtained from Sigma UK.

2.3. Growth studies

10^6 sporidia ml^{-1} were inoculated into 1 l liquid YEPD in 2-l flasks. At time zero, triadimenol was added at the dose identified as the MIC of ATCC 14826. At various times 100 ml YEPD liquid culture was removed for sterol extraction, cell counts and dry cell weight determination.

2.4. Sterol extractions

Extraction of non-saponifiable sterols followed the method outlined by Woods [14] with sporidia saponified at 90°C for 1 h with 3 ml MeOH, 2 ml 60% KOH and 2 ml 0.5% pyrogallol in MeOH. Extraction was completed with 3×5 ml hexane extractions and evaporated to dryness under N_2.

Following silylation for 1 h at 60°C with BSTFA (20 μl) in 50 μl of toluene, sterols were analysed by GC-MS (VG 12-250 (VG BIOTECH)) using split injection with a split ratio of 20:1. Sterol identification was by reference to the literature [15–18] for relative retention time (RRT) values and mass spectra.

3. Results and discussion

The mechanisms of azole-induced growth arrest have been characterised in *S. cerevisiae*, with arrest

Table 1
Percentage sterol compositions of *U. maydis* after treatment with the azole fungicide triadimenol at the minimum inhibitory concentration [a]

	Percentage sterol composition							
	0 h	3 h	6 h	9 h	12 h	18 h	24 h	24 h [b]
Ergosta-tetraenol	3.6	–	–	1.8	2.0	–	–	2.3
Ergosta-8,22-dienol	0.6	–	–	–	–	–	–	2.7
Ergosterol	51.6	30.6	28.6	18.2	12.9	10.7	6.0	55.6
Ergosta-7,22-dienol	–	–	–	–	–	–	–	4.2
14α-Methyl fecosterol	3.2	–	–	11.8	12.3	11.6	17.8	–
Ergosta-5,7-dienol	27.4	4.9	11.2	7.8	5.1	5.5	3.1	28.2
Ergost-7-enol	5.6	–	–	0.2	–	–	2.5	2.3
Obtusifoliol	–	14.6	11.9	25.9	32.4	23.8	33.4	0.5
14α-Methyl-3,6-diol [c]	–	–	–	–	–	5.6	5.8	0.5
Eburicol	1.9	43.7	42.4	31.2	34.0	36.6	29.3	1.9
Unidentified sterols	6.1	6.2	5.9	3.1	1.3	6.2	2.1	1.1

[a] 1×10^{-6} M.
[b] Untreated control.
[c] 14α-methylergosta-8,24(28)-diene-3β,6α-diol.

associated with the accumulation of 14α-methyl-3,6-diol (the proposed product of attempted 5(6) desaturation of 14α-methylfecosterol) [2]. In *U. maydis* ATCC 14826 the involvement of this diol in growth arrest was considered unlikely as its accumulation occurred after growth was arrested (Table 1). Total concentration of 14α-methyl-3,6-diol remained low relative to *S. cerevisiae* and was not observed before the point of growth arrest.

In order to examine what mechanism(s) of azole resistance occur in *U. maydis*, direct selection of azole resistant mutants was employed through screening a wild-type ATCC 14826 population treated at a triadimenol concentration five-fold above the MIC dose. A mutant (B3) was found to have a sterol phenotype characteristic of a leaky lesion in the sterol $\Delta^{5(6)}$desaturase enzyme, as indicated by abnormally high levels of D7 sterols (10.7% ergosta-7-enol and 21.0% ergosta-7,22-dienol) for this strain (Table 2). B3 was shown to exhibit cross-resistance to azole antifungals. MICs observed for ATCC 14826 and B3 were: for triadimenol, 1 μM and 50 μM; for diclobutrazol, 0.5 μM and 10 μM; for prochloraz, 10 μM and 120 μM; for tebuconazole, 0.1 μM and 1 μM; and for fenarimol (a pyrimidine) 5 μM and 50 μM, respectively. Mutant B3 was found to be equally sensitive to the polyene, amphotericin B, when compared to ATCC 14826 with a MIC of 1.0 μg ml^{-1} which reflects the retention of significant quantities of ergosterol in this mutant.

The effect of triadimenol treatment on B3 was examined over 24 h using the dose observed as the MIC for the parent strain (Table 2). Azole inhibition of the sterol P450 $_{14\alpha\text{-dm}}$ was rapid, as shown by the accumulation within 3 h of treatment of substrate (eburicol) and the decrease in ergosterol and other desmethyl sterols. Accumulation of 14α-methyl sterols, with a consequential decrease in desmethyl sterols, continued throughout the treatment period. The accumulation of 14α-methylfecosterol up to 32.6% of the total sterols after 24 h, combined with the very low levels of 14α-methyl-3,6-diol (4.7%), compared to ATCC 14826's levels of 18.2% and 14.2% for each sterol, respectively. This again indicated the reduced activity for the $\Delta^{5(6)}$desaturase enzyme in B3, reflected in the poor conversion of 14α-methylfecosterol into 14α-methyl-3,6-diol.

The growth rate of B3 was unaltered compared to ATCC 14826 (Fig. 2a). However, the growth rate for B3 under azole treatment was observed to slow between the 9th and 12th hour when the growth of ATCC 14826 stopped (Fig. 2b). At this time, ergosterol and 14α-methylfecosterol concentrations were low (6.6% and 5.2% for B3, 9.2% and 12.7% for ATCC 14826). With triadimenol treatment of ATCC 14826, growth arrest at the MIC occurred once the

Table 2
Effects on relative sterol compositions and growth (dry cell weights and cell counts) of B3 treated at the triadimenol minimum inhibitory concentration [a] of ATCC 14826

	Percentage sterol composition							
	0 h	3 h	6 h	9 h	12 h	18 h	24 h	24 h [b]
Ergosta-tetraenol	4.9	Trace	Trace	–	–	–	–	2.7
Ergosterol	47.2	23.4	14.5	6.6	5.2	3.4	2.7	41.5
Ergosta-7,22-dienol	21.0	–	–	–	–	–	–	20.8
14α-Methylfecosterol	–	3.6	7.1	9.2	12.7	23.6	32.6	–
Ergosta-5,7-dienol	5.2	–	–	–	–	–	–	17.1
Ergosta-7-enol	10.7	–	–	–	–	–	–	3.9
4-Methylergosta-8,24-dienol	2.5	–	–	–	–	–	–	1.1
Obtusifoliol	–	6.3	17.7	18.4	21.7	24.1	24.6	–
Eburicol	3.9	66.7	60.7	65.8	60.5	41.9	31.0	1.7
Lanosterol	4.5	–	–	–	–	–	–	10.0
14α-Methyl-3,6-diol [c]	–	–	–	–	–	2.6	4.7	–
Unknown sterols	–	–	–	–	–	4.4	4.4	8.4

[a] 1×10^{-6} M.
[b] Untreated 24 h control.
[c] 14α-methylergosta-8,24(28)-diene-3β,6α-diol.

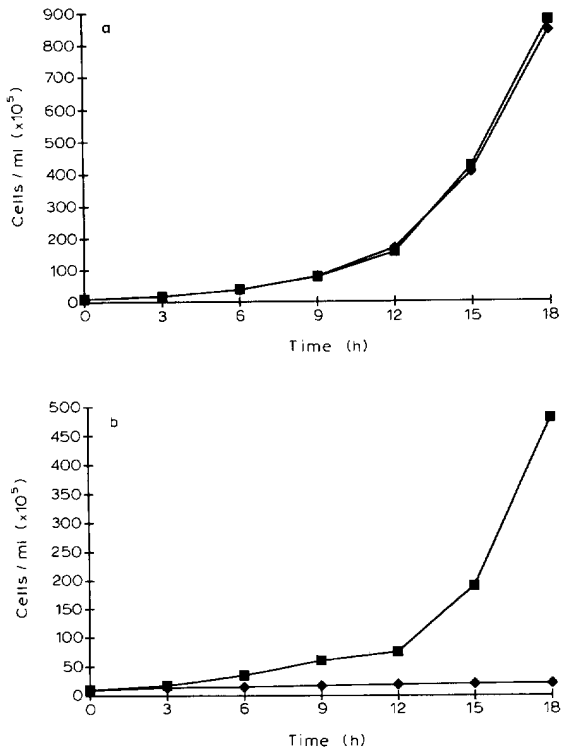

Fig. 2. (a) Growth rate of B3 (■) in comparison to ATCC 14826 (◆). (b) Effect on growth of the mimimum inhibitory concentration of triadimenol (1×10^{-6} M) on ATCC 14826 (◆) and B3 (■).

combined proportions of these two sterols fell below 20%. As time increased past this point, the growth rate of B3 increased, probably as a result of the accumulation of 14α-methylfecosterol to levels above 20% of total sterols.

Although B3 was observed to be resistant to azole, higher concentrations could cause growth inhibition. Triadimenol treatment of B3 at its MIC, rather than at the MIC for ATCC 14826, resulted in a sterol profile consisting of 93.6% 14α-methyl sterols of which 53.3% represented 14α-methylfecosterol (Table 3). The reason for the accumulation of higher levels of 14α-methylfecosterol at the higher azole dose was unclear. The level of functional sterol (14α-methylfecosterol) observed for this treatment might be expected to support fungal growth and implied a secondary antifungal mode of action of azole at this higher dose.

The spontaneous mutation frequency observed for the occurrence of mutant B3 was 10^{-8} which, together with the sterol phenotype, is consistent with a single mutation conferring azole resistance. In *S. cerevisiae* a similar leaky $\Delta^{5(6)}$desaturase mutant was isolated [7] and our studies here confirm the general relevance of the framework of mode of action and resistance established for *S. cerevisiae*. Many of the sterol $\Delta^{5(6)}$desaturase mutants isolated in *S. cerevisiae* had stringent blocks, but recently gene disrup-

Table 3
Comparison of the relative sterol compositions of ATCC 14826 and B3 under various triadimenol concentrations

Sterol	ATCC 14826 [a]	B3 [a]	ATCC 14826 [b]	B3 [b]	B3 [c]
Ergosta-tetraenol	2.3	4.9	–	–	–
Ergosta-8,22-dienol	2.7	–	–	–	–
Ergosterol	55.6	47.2	1.9	2.7	6.4
Ergosta-7,22-dienol	4.2	21.0	–	–	–
14α-Methylfecosterol	–	–	18.2	32.6	53.3
Ergosta-5,7-dienol	28.2	5.2	0.3	–	–
Ergosta-7-enol	2.3	10.7	1.9	–	–
4-Methylergosta-8,24-dienol	–	2.5	–	–	–
Obtusifoliol	0.5	–	38.3	24.6	15.7
Eburicol	1.9	3.9	23.5	33.9	18.4
Lanosterol	–	4.5	–	–	3.2
3,6-Diol	–	–	14.2	7.3	3.0
Unidentified sterols	1.6	0.1	0.1	8.4	0.0

[a] Untreated.
[b] Treated at 1×10^{-6} M triadimenol.
[c] Treated at 1×10^{-5} M triadimenol.

tion studies indicated active sterol $\Delta^{5(6)}$desaturase was required for aerobic growth [19]. Whether mutants completely blocked in sterol $\Delta^{5(6)}$desaturase could be isolated in petite negative fungi remains to be determined and it may only be possible to isolate leaky sterol $\Delta^{5(6)}$desaturase mutants. As such mutants can contain significant quantities of ergosterol, superficial characterisation may identify them as wild-type for sterol biosynthesis. Attention to the proportion of sterols is essential in future studies on azole resistance screening of clinical or field isolates.

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