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Altered P450 activity associated with direct selection for fungal azole resistance

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Abstract Azole antifungals inhibit CYP51A1-mediated sterol 14α-demethylation and the mechanism(s) of resistance to such compounds in *Ustilago maydis* were examined. The inhibition of growth was correlated with the accumulation of the substrate, 24-methylene-24,25-dihydrolanosterol (eburicol), and depletion of ergosterol. Mutants overcoming the effect of azole antifungal treatment exhibited a unique phenotype with leaky CYP51A1 activity which was resistant to inhibition. The results demonstrate that alterations at the level of inhibitor binding to the target site can produce azole resistance. Similar changes may account for fungal azole resistance phenomena in agriculture, and also in medicine where resistance has become a problem in immunocompromised patients suffering from AIDS.

Key words: Cytochrome P450; Azole; Resistance

1. Introduction

Cytochrome P450s (CYPs) are a superfamily of monooxygenases involved in various biosynthetic functions as well as being central to the initial metabolism of xenobiotics [1]. Fungi possess a typical eukaryotic CYP system and the predominant P450 of vegetative yeast cells has been observed to be sterol 14α-demethylase (CYP51A1), an enzyme of the ergosterol biosynthetic pathway [2]. CYP51A1 has been observed to 14α-demethylate the sterol substrate via three sequential hydroxylations [3] and has become recognised as the target of the azole antifungals employed in medicine and agriculture [4]. The azoles inhibit fungal CYP51A1 selectively, binding to the haem as a sixth ligand with the N-1 substituent group interacting with the apoprotein [5]. The interaction with the apoprotein determines the selectivity, although these compounds can inhibit other CYPs at higher concentrations including plant and animal sterol 14α-demethylases [6].

Resistance to antimicrobial drugs and pesticides is a common observation with widespread use, but problems with the azole antifungals emerged first in agriculture [7] and subsequently in clinical situations [8]. For the latter the requirement for lifetime maintenance for patients suffering AIDS, and who usually suffer fungal infections, may have increased the probability of fungal resistant mutants being selected. Fungal infection is also common following therapy after organ transplantation or chemotherapy where the immune system is suppressed.

Studies on the model organism *Saccharomyces cerevisiae* have provided the most comprehensive data on fungal sterol requirements and azole resistance. CYP51A1 inhibition results in the accumulation of substrate (lanosterol) and reduction in ergosterol. However, lanosterol has been observed to be subject to subsequent metabolism, resulting in the removal of the C4-methyl groups, methylation at C24 and 6-hydroxylation by sterol $\Delta^{5,6}$ desaturase to produce 14α-methylergosta-8,24(28)-dien-3β,6α-diol (14αmethyl-3,6-diol) (Fig. 1) [9]. This sterol accumulated to a maximum at the point of growth arrest and the presence of the 6-hydroxy group in this sterol is thought to interfere with sterol/phospholipid hydrogen bonding in the membrane.

The predominant mechanism of azole resistance for *S. cerevisiae* is through a block in sterol $\Delta^{5,6}$ desaturase activity [10]. This results instead in the accumulation of 14α-methylfecosterol which can fulfill requirements for growth. Mutant *S. cerevisiae* strains lacking sterol 14α-demethylase activity are also azole resistant [11]. However, complementation studies using plasmid-bourne sterol 14α-demethylase have shown resistance can be attributed to the suppressor mutations in sterol $\Delta^{5,6}$ desaturase which allow the aerobic growth of such mutants [4].

Alteration of the target CYP51A1 has been proposed previously as a possible route to resistance to azole antifungals [4]. Such azole resistant mutants have been obtained here from the phytopathogen *U. maydis*. They show a significant difference in their mechanism of resistance based on an alteration in the target CYP51A1 activity to confer reduced inhibitory effect of azole antifungals.

2. Materials and methods

2.1. Chemicals

Unless otherwise indicated all chemical reagents were supplied by Sigma U.K. Azoles, [^{14}C]Triadimenol and fenarimol (a pyrimidine sterol 14α-demethylase inhibitor) were supplied by Long Ashton Research Station, Bristol University, UK. [^{14}C]Mevalonic acid was obtained from Amersham.

2.2. Culture conditions

U. maydis strain ATCC 14826 was used. Growth was supported on 1% (w/v) Difco yeast extract, 2% (w/v) Difco peptone and 2% (w/v) glucose (YEPD) liquid medium at 25°C and 150 rpm or on solid YEPD plates using 2% (w/v) Difco Bacto-Agar.

2.3. Antifungal studies

Unless otherwise specified, all azoles were supplied by Long Ashton Research Station. Prochloraz, tebuconazole and the pyrimidine, fenarimol were dissolved in methanol to a stock concentration of 10^{-2} M, while hexaconazole, triadimenol and diclobutrazol (Zeneca Agrochemicals) were dissolved at the same concentration in dimethyl sulphoxide (DMSO). Amphotericin B (Sigma UK) and cycloheximide (Sigma UK) were dissolved in DMSO to a final stock concentration of

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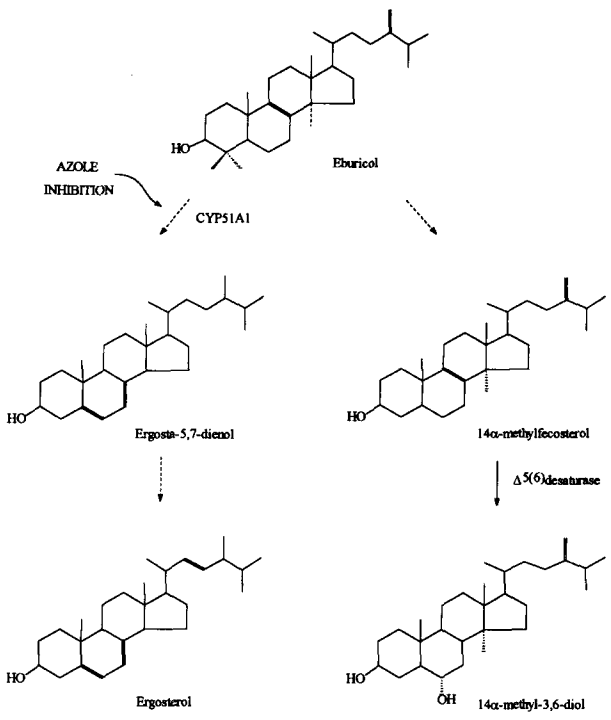

Fig. 1. Pathway of sterol formation and alternative pathway after azole antifungal treatment.

1.08×10^{-3} M. Minimum inhibitory concentrations (MIC) were determined for sporidia harvested in late log phase by centrifugation at 4000 rpm and inoculation into 2 ml YEPD medium to give a final concentration of 10^6 cells · ml^{-1}. Triplicate treatments using azole and pyrimidine doses between 1×10^{-4} M and 5×10^{-8} M, and amphotericin B and cycloheximide doses between 5.4×10^{-5} M and 1.08×10^{-8} M, and between 2×10^{-6} M and 2×10^{-9} M, respectively were used. Test MIC cultures were incubated for 24 h and 72 h at 25°C and 150 rpm with growth assessed by cell counts (haemocytometer), and by colony forming units per ml.

2.4. ATCC 14826 growth study

Experiments to determine the effect of treatment with time when using the MIC of triadimenol were conducted under identical conditions to those described above except 1 litre cultures in 2 litre flasks were employed, with 100 ml of culture harvested for sterol analysis, dry cell weight and cell count determinations.

2.5. Sterol isolation and analyses

Extraction of non-saponifable sterols followed the method outlined by Woods et al. [12] with GC-MS analyses conducted as described by Loeffler et al. [13]. Peak analysis and identification were realised through reference to current literature [13–16] for relative retention time (RRT) values and fragmentation ion patterns.

2.6. Cell free sterol biosynthesis analyses

Assay of azole inhibition of ergosterol biosythesis was using the cell free biosynthesis system described extensively in the literature [17,18]. 500 ml flasks containing 200 ml YEPD were inoculated with strains to a concentration of 10^6 sporidia per ml and grown at 25°C at 150 rpm. Cells were harvested in late log phase by centrifugation (4000 rpm for 10 min) and resuspended in 25 ml 0.2 M phosphate buffer containing 140 ml 0.2 M KH_2PO_4, 360 ml 0.2 M K_2HPO_4, 20% (v/v) glycerol and 1 mM EDTA (pH 6.8). Cell disruption was achieved using a Braun MSK homogeniser with three 30 s disruptions. Cell free extracts were harvested by centrifugation at 4000 rpm for 10 min. 0.5 ml of co-factor mix (20 mg NADPH, 18 mg NAD, 17 mg NADP, 55 mg ATP, 20 mg glutathione, 30 mg glucose-6-phosphate dissolved in 1 ml phosphate buffer) was added to 9.5 ml of supernatant with 20 μl 1 M magnesium chloride and 10 μl 1 M manganese chloride. To 1 ml of the above mix triadimenol was added at varying concentrations. 10 μl [^{14}C]mevalonic acid (specific activity 53 mCi/mmol) was added to each sample and incubated at 25°C for 2 h. Sterol extraction of non-saponifiable sterols was conducted as described above.

Sterol identification was achieved through thin layer chromatography (Merck) with solvent as a 4:1 mix of cyclohexane:diethyl ether with ergosterol and lanosterol co-run as standards. Autoradiograms were developed over 7 days at −80°C and radioactivity incorporated into ergosterol quantified on a Beckman LS 1801 scintillation counter.

2.7. Preparation of microsomal fraction

Sporidia of *U. maydis* strain ATCC 14826 and Tri^{R-1}, Tri^{R-2} and Tri^{R-3} in the logarthmic phase of growth were inoculated (10^6 cells · ml^{-1}) into liquid YEPD (1 litre) in 2 litre flasks and incubated at 25°C and 110 rpm until a cell density of 5×10^7 cells · ml^{-1} was reached. Preparation of the microsomal fraction from protoplasts of *U. maydis* followed the procedure outlined by Carelli et al. [19]. Protein content was estimated using the procedure outlined by Lowry et al. [20].

2.8. Accumulation of triadimenol

Cells were inoculated into 100 ml YEPD in 250 ml flasks at 10^6 cells/ml and incubated at 25°C and 150 rpm until growth reached stationary phase. Cells were harvested by centrifugation at 4000 rpm, washed in phosphate buffer, pH 7.0 (phosphate buffer tablets pH 7.0, BDH) and resuspended in 1 ml phosphate buffer, pH 7.0 at a final concentration of 2×10^9cells/ml. [^{14}C]Triadimenol was added to a concentration of 1×10^{-5} M from a 22.2 μCi/mg stock and incubated for 1 h at 25°C and shaken every 10 min. Cells were harvested and washed in 5 ml cold triadimenol (5×10^{-5} M). Cells were re-harvested onto Whatman no.1 filters by vacuum filtration. This wash was repeated

Table 1
Percentage sterol compositions of *U. maydis* ATCC 14826 after treatment with the azole fungicide triadimenol at the MIC[a]

Sterol	Time (hours)											
	0 ct[b]	0	0.5	1	3	6	9	12	18	24	36	36 ct[b]
Ergostatetraenol	3.6	3.5	3.0	2.9	–	–	1.8	2.0	–	–	–	2.3
Ergosta-8,22-dienol	0.6	1.0	1.6	1.7	–	–	–	–	–	–	1.9	2.7
Ergosterol	51.6	55.0	39.9	39.9	30.6	28.6	18.2	12.9	10.7	6.0	1.7	55.6
Ergosta-7,22-dienol	–	1.0	2.2	1.5	–	–	–	–	–	–	–	4.2
14α-Methyl fecosterol	3.2	1.2	1.9	–	–	–	11.8	12.3	11.6	17.8	18.2	–
Ergosta-5,7-dienol	27.4	25.8	17.8	18.2	4.9	11.2	7.8	5.1	5.5	3.1	0.3	28.2
Ergost-7-enol	5.6	6.0	3.0	–	–	–	0.2	–	–	2.5	1.9	2.3
Obtusifoliol	–	–	3.8	5.8	14.6	11.9	25.9	32.4	23.8	33.4	38.3	0.5
14α-Methyl-3,6-diol[c]	–	–	–	–	–	–	–	–	5.6	5.8	14.2	0.5
Eburicol	1.9	2.2	21.8	25.6	43.7	42.4	31.2	34.0	36.6	29.3	23.5	1.9
Unidentified sterols	6.1	4.3	5.0	4.2	6.2	5.9	3.1	1.3	6.2	2.1	0.1	1.1
Dry cell weight (mg)	54	56	55	58	65	85	90	100	100	100	100	296

Key: [a] = 1×10^{-5} M; ct[b] = untreated control; [c] = 14α-methylergosta-8,24(28)-diene-3β,6α-diol.

Table 2
Comparison of minimum inhibitory concentrations for various antifungals in parent and mutant isolates

Drug type	ATCC 14826	TRI^{R-1}	TRI^{R-2}	TRI^{R-3}
Azoles (μM)				
Diclobutrazol	1	10	50	50
Triadimenol	1	5	10	10
Tebuconazole	0.1	0.5	1	1
Procholaz	10	50	100	50
Hexaconazole	1	10	50	50
Other (μM)				
Fenarimol	5	10	30	50
Amphotericin B	0.6	0.9	1.1	1.1
Cycloheximide	0.2	0.2	0.2	0.2

three times. Cells and filters were baked at 80°C until dry. Counts per minute were measured on a Beckman LS 1801 scintillation counter. Cellular content was calculated in triplicate as μmoles triadimenol/10^9cells.

3. Results

3.1. Analysis of triadimenol induced growth arrest of U. maydis

U. maydis treated with triadimenol has been employed here as a comparative experimental system to investigate the general relevance of 14α-methyl-3,6-diol to azole induced growth arrest. Treatment of this phytopathogen with the minimum inhibitory concentration of triadimenol resulted in an overall accumulation of 14α-methyl sterols with a concomitant decrease in the desmethyl sterols (Table 1) which is in agreement with previous studies [14,21].

Ergosterol concentration was observed to decrease across the treatment period from an initial level of 55% to a final concentration of 1.7% at 24 h. Similar decreases of other desmethyl sterols were also observed such as that of ergosta-5,7-dienol. Azole treatment resulted in the accumulation of 14α-methyl sterols, predominately eburicol and obtusifoliol. Eburicol content rose sharply from an untreated level of 2.2% of total sterol to a peak of 43.7% at the third hour sample, whereafter the level decreased to 23.5% of total sterol at 36 h. Obtusifoliol showed increased accumulation across the treatment study, rising from 0% to 38.3% of total sterol at the thirty-sixth hour sample. 14α-Methyl-3,6-diol remained undetected until the eighteenth hour, and thereafter increased upto a final relative concentration of 14.2%.

Growth arrest of ATCC 14826 occurred by the twelfth hour as shown by dry cell weight and cell counts (Table 1). At this point the concentration of ergosterol was 12.9%, eburicol and obtusifoliol were at 34.0% and 32.4%, respectively, and 14α-methyl-3,6-diol was not observed.

3.2. Isolation of azole resistant mutants

Screening for mutants used 10^6 cells ATCC 14826 on 20 ml solid YEPD in 9 cm petri dishes. Triadimenol concentrations of five- and ten-fold above the MIC of ATCC 14826, yielded three triadimenol resistant mutants with a frequency of 10^{-8}. Cross-resistance to the azoles diclobutrazol, hexaconazole, procholaz and tebuconazole and the pyrimidine fenarimol was observed for the mutants Tri^{R-1}, Tri^{R-2} and Tri^{R-3} at varying levels (Table 2). Sensitivities to other classes of antifungal drugs were investigated and for amphotericin B and cycloheximide similar sensitivities to values for ATCC 14826 were observed. ATCC 14826 and the azole mutants all grew at the same rate with a doubling time of 2.5 h when untreated.

3.3. Cellular content of [^{14}C]triadimenol

In order to examine the possible involvement of uptake and/or efflux mechanisms in the azole resistance of these mutants, the cellular content of triadimenol was examined. From triplicate treatments similar values of uptake for each mutant and the parent strain were observed (Table 3).

3.4. Sterol analysis of Tri^{R-1}, Tri^{R-2} and Tri^{R-3}

Sterol profiles of each mutant were determined in the absence of azole (Table 4). The relative percentage compositions of 14α-methyl sterols were 62.2%, 80% and 49.9% and for ergosterol were 29.1%, 16.1% and 39.7% for Tri^{R-1}, Tri^{R-2} and Tri^{R-3} respectively compared to the 14α-methyl sterol content of 5.1% and an ergosterol relative concentration of 55.0% for ATCC 14826. Under triadimenol treatment for 24 h at the MIC dose for ATCC 14826, the relative percentage compositions of ergosterol decreased in Tri^{R-1} (8.2%) and Tri^{R-3} (31.2%). A greater reduction was observed when treating with the MIC of each mutant, where the relative concentration of ergosterol was 3.6% and 10.2%, respectively (Table 4). Tri^{R-2} showed little variation in ergosterol content under treatment or total 14α-methyl sterol concentrations at either treatment dose (Table 4).

3.5. Levels of sterol $P450^{14\alpha\text{-}dm}$ from ATCC 14826, Tri^{R-1}, Tri^{R-2} and Tri^{R-3}

The microsomal fractions produced a maximum at 448 nm in reduced carbon monoxide difference spectra for ATCC 14826 and each mutant similar to that reported previously for *U. maydis* [19]. Specific contents of 20(±0.3) pmol·mg^{-1}, 21(±0.5) pmol·mg^{-1}, 20(±1.0) pmol·mg^{-1} and 20(±0.3) pmol·mg^{-1} for ATCC 14826, Tri^{R-1}, Tri^{R-2}, and Tri^{R-3} were determined, respectively (Table 3).

3.6. Inhibition of sterol $P450^{14\alpha\text{-}dm}$ activity in vitro

The in vitro sterol biosynthesis for parent and mutants were compared under triadimenol treatment. IC_{50} (concentration required to inhibit ergosterol biosynthesis by 50%) was determined by following ergosterol biosynthesis from [^{14}C]mevalonic acid [17]. The sterol 14α-demethylase of each mutant was observed to be resistant (Table 3). Tri^{R-1} required five fold more triadimenol and Tri^{R-2} and Tri^{R-3} required ten fold the concentration of triadimenol for equivalent inhibition to that observed for ATCC 14826.

Table 3
Comparison of untreated growth rates, cellular content of [^{14}C]triadimenol, specific contents of P450 and IC_{50} values for in vitro inhibition of ergosterol biosynthesis by triadimenol in parent and mutant isolates

	ATCC 14826	Tri^{R-1}	Tri^{R-2}	Tri^{R-3}
μmol [^{14}C]triadimenol/10^9 cells	12 ± 0.5	13.5 ± 0.1	14 ± 0.3	12 ± 0.1
pmol P450/mg microsomal protein	20 ± 0.3	21 ± 0.5	20 ± 1.0	20 ± 0.3
IC_{50} (μM)	0.08 ± 0.003	0.3 ± 0.03	0.8 ± 0.03	0.8 ± 0.03
Doubling time (h)	2.5	2.5	2.5	2.5

Table 4
Sterol profiles of ATCC 14826 in comparison to mutant isolates

Sterol	Relative percentage sterol composition									
	ATCC 14826	TRI^{R-1}	TRI^{R-1a}	TRI^{R-1b}	TRI^{R-2}	TRI^{R-2a}	TRI^{R-2c}	TRI^{R-3}	TRI^{R-3a}	TRI^{R-3b}
Ergosta-tetraenol	2.8	7.9	–	–	0.7	1.4	Trace	5.0	Trace	Trace
Ergosterol	55.6	29.1	8.2	3.6	16.1	22.9	20.2	39.7	31.2	10.2
14α-Methylfecosterol	–	20.8	7.1	50.7	15.3	5.5	5.7	10.3	11.6	32.4
Ergosta-5,7-dienol	28.2	8.6	–	–	–	5.3	6.4	5.7	9.9	–
Obtusifoliol	–	21.7	18.5	17.4	31.0	18.8	14.4	29.4	24.1	19.2
14α-Methyl-3,6-diol[d]	0.5	1.8	–	5.5	19.5	20.0	21.8	–	2.1	4.9
Eburicol	1.9	1.8	66.2	22.8	15.5	24.1	27.5	4.9	20.4	33.2
$M^+ = 552$	–	–	–	–	–	–	–	4.9	–	–
Unidentified sterols	11.0	8.3	0.0	0.0	6.8	2.0	4.0	0.1	0.6	0.0

Key: [a] = 1×10^{-6} M triadimenol; [b] = 5×10^{-6} M triadimenol; [c] = 1×10^{-5} M triadimenol; and [d] = 14α-methylergosta-8,24(28)-diene-3β,6α-diol.

4. Discussion

CYP51A1 is essential for aerobic growth of yeast [22], and probably in fungi generally. Studies on this protein and its inhibition are of practical and academic significance and enantiomers of the numerous drugs and pesticides are proving useful tools with which to undertake molecular modelling studies [4,23]. The discovery here that mutations in CYP51A1 can result in reduced inhibition by azole antifungals will provide further important information with which to understand the active site topology.

The consequences of inhibition of the enzyme is the reduction of ergosterol and accumulation of 14α-methylated sterols. However, one 14α-methylated sterol does satisfy the growth requirements of *S. cerevisiae*. *14α*-Methylfecosterol accumulates in sterol $\Delta^{5,6}$ desaturase mutants under azole treatment instead of 14-methyl-3,6-diol [10] and results in azole resistance. This mechanism of resistance was not apparent in the *U. maydis* studies reported here in which mutants were challenged directly with azole antifungal, but a possible leaky sterol $\Delta^{5(6)}$ desaturase mutant which is azole resistant is the subject of further investigation. An examination of the selective pressure under which the *U. maydis* was placed indicated the period of growth arrest occurred without accumulation of 14-methyl-3,6-diol.

The frequency with which sterol $\Delta^{5,6}$ desaturase mutations and azole resistance arises in *S. cerevisiae* is approximately 10^{-5} (unpublished observations). However, resistant mutants occurred here with a frequency of approximately 10^{-8} suggesting mutagenesis at a limited number of nucleotide positions could cause resistance. The biochemical characterisation of the azole resistant mutants showed they were not associated with degradation of the antifungal or with altered uptake or efflux. The latter has been associated with resistance in some strains of *Candida albicans* [24] and *Penicillium italicum* [25]. Similarly, the specific content of P450 remained unaltered in each mutant in comparison to the parent strain, ATCC 14826. This indicated that resistance was not as a result of increased P450 expression, which has previously been linked with azole resistance in tobacco [26].

The lesion(s) resulting in resistance in the three mutants of *U. maydis* was identified as being in *CYP51A1*. The mutants showed altered patterns of sterol biosynthesis in vivo with the accumulation of increased quantities of the CYP51A1 substrate, 24-methylene-24(25)-dihydrolanosterol. Coupled to the observation of leaky CYP51A1 activity was the observation that in vitro inhibition of sterol 14α-demethylase in the mutants required increased concentrations of azole antifungal when compared to the wild-type. Together this information indicates a change in the apoprotein of CYP51A1 altering the activity, but reducing the ability of azole antifungals to inhibit the enzyme. Other possible changes which could produce a leaky sterol 14α-demethylase would not account for the relative resistance of the in vitro activity to azole inhibition.

Ergosterol was also at a reduced level in all the mutants, but not such that growth was altered (Table 3). An important factor concerning resistant strains found in agriculture is the requirement for robust growth under environmental conditions and the phenotype of the mutants studied here would seem to satisfy this requirement. As the mutants still contain ergosterol superficial classification might also identify them as being wild-type. Examination of azole resistance in agricultural, or clinical isolates, should be made with this information in mind and detailed biochemical investigation may be needed to confirm an alteration in *CYP51A1*. Our efforts are now concerned with isolating *CYP51A1* from wild-type and azole resistant mutants. Nucleotide sequence comparison and site-directed mutation studies will be required to confirm the alterations in the protein causing resistance and should provide important information to assist our understanding of the structure of this protein and possible routes to combat resistance.

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