 Mutation in cytochrome P-450-dependent 14α-demethylase results in decreased affinity for azole antifungals

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The conversion of lanosterol into ergosterol, the main sterol in most pathogenic yeasts and fungi, is a complex multistep process, catalysed by enzymes in the mitochondria and endoplasmic reticulum [1]. A key step in this segment of the sterol biosynthetic pathway is the cytochrome P-450 (P450)-dependent removal of the methyl group (C-32) at C-14. Several imidazole derivatives are inhibitors of P450-dependent enzymes of insects and rats [10]. As shown in Table 1, similar results are obtained with the orally active ketoconazole (imidazole) and itraconazole (triazole). Compared with the other orally active azoles, the triazole-substituted antifungal, fluconazole, shows a much lower affinity (Table 1).

Effects of azole antifungals on P450 (s) and P450-dependent sterol synthesis

Several imidazole derivatives are inhibitors of P450-dependent enzymes of insects and rats [10]. They interact with P450, yielding type II spectra. These spectra (maximum absorption peak at about 430 nm and a trough at about 390 nm) are found with compounds that bind to the oxidized form of the haem iron ion.

The imidazole antifungal, micafungazole, added to microsomal suspensions of S. cerevisiae and C. albicans also yielded type II spectra [11]. This indicates that the unsaturated nitrogen (N-3) of the imidazole moiety binds to the haem iron ion opposite to the thiolate binding, i.e. the site of oxygen binding (the sixth co-ordination place). Further evidence is derived from the competition of miconazole with CO for binding to the haem iron ion. When miconazole is added to a suspension of yeast microsomes, before reduction of the haem iron and saturation with CO, a 50% decrease in absorption at 448 nm (absorption maximum of the reduced CO-yeast P450 complex) is achieved at 8.6 x 10^-4 m (S. cerevisiae) and 9.3 x 10^-4 m (C. albicans).

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Yoshida [3] studied the interaction of ketoconazole with purified P450(C). Binding of ketoconazole to this P450 is quantitative and the Kd is assumed to be less than 10^-8 m. Yoshida & Aoyama [14, 15] also proved that ketoconazole inhibits lanosterol 14α-demethylation by a reconstituted system consisting of P450(C) and NADPH-P450 reductase, both purified from S. cerevisiae microsomes. Complete inhibition by ketoconazole is achieved at the concentration which is equivalent to the P450(C) concentration, i.e. 1.4 x 10^-7 M. Ketoconazole does not inhibit NADPH-cytochrome c reductase activity catalysed by the P450

Abbreviations used: P450, cytochrome P-450; P450(C), cytochrome P-450-dependent 14α-demethylase; P450(14DM), cytochrome P-450 from Saccharomyces cerevisiae SG1; IC50, concentration producing 50% inhibition; DMSO, dimethylsulphoxide; CYG medium, casein hydrolysate/yeast extract/glucose medium; K-14, 148 passages in CYG medium containing 10^-5 M-ketoconazole dissolved in dimethylsulphoxide; C-148, 148 passages in CYG medium containing dimethylsulphoxide.
reductase. These results, together with the fact that ketoconazole and other imidazole and triazole derivatives induce marked spectral changes of the purified P450, prove that these azole derivatives inhibit the 14α-demethylation by a direct effect on the P450d10m [13].

The effect of azole antifungals on the P450-dependent ergosterol synthesis has been proven in many yeasts and fungi. Examples are C. albicans, C. hortianum, Aspergillus spp. and Penicillium spp. [11, 16, 17]. Recently, we were able to prove that both ketoconazole and itraconazole inhibit ergosterol synthesis in Histoplasma capsulatum (yeast form). Depletion of ergosterol is already achieved after 48 h of growth in the presence of 10−7 M-ketoconazole and 3 × 10−8 M-itraconazole [H. Vanden Bossche, unpublished work]. As can be expected from the above discussed interaction of azole antifungals with P450d10m, inhibition of ergosterol synthesis coincided with the accumulation of sterols with a methyl group at C-14. Examples are 14-methyl-△2,4-20-ergosta-diene-3β,6α-diol, 14-methyl-fecosterone, 14-methyl-△2,3,5-ergosta-tetraene-3β-ol, obtusifoliol, obursifoliol, 24-methylenedihydrofumosterol and lanosterol.

Resistance to azole antifungals

Yoshida et al. [18] purified a P450 (P450d10m) from a nystatin-resistant mutant, S. cerevisiae SG1, which is defective in lanosterol 14α-demethylation. A single nucleotide change resulting in substitution of aspartic acid for glycine-310 of P450d10m was found to have occurred in P450d10m [6]. In this protein the 6th ligand to the haem iron is a histidine residue [6] instead of the hydroxyl group of water or of a serine, tyrosine or threonine residue, which is the most likely candidate for the 6th ligand in normal P450s. This P450d10m not only lost its catalytic activity, but also did not interact with the pyridyl antifungal, buthiobate [19]. Hence, the field strength of the native sixth ligand (histidine) is stronger than that of pyridine. This S. cerevisiae mutant is also less sensitive to ketoconazole and itraconazole. The concentration producing 50% inhibition (IC50) was 2 × 10−5 M-ketoconazole, whereas, with the parent strain (DS87), this inhibition was already achieved at 7 × 10−8 M. Itraconazole did not affect growth of SG1 at concentrations up to 5 × 10−4 M (i.e. at the limit of solubility). The IC50 value for the growth of strain DS87 was 1.9 × 10−5 M.

To measure the affinity of ketoconazole and itraconazole for the microsomal P450s of both the parent strain (DS87) and of the SG1 mutant, increasing concentrations (up to 5 × 10−6 M) of ketoconazole or itraconazole were added to microsomal suspensions containing P450s in their oxidized form. The controls contained the same amount of solvent (dimethylsulphoxide; DMSO). Upon addition of the reduc- tant, and saturation with CO, maximal absorption at 447 nm (SG1) and 449 nm (DS87) were observed under control conditions. As shown in Fig. 1, these reduced P450-CO complexes were stable over the 60 min measurement period. Both itraconazole and ketoconazole decrease the absorption at 447 nm (SG1) and 449 nm (DS87). The P450d10m-itraconazole complex is less stable than that formed with the micro- somal P450d10m of the parent strain. However, as can be deduced from the gradual spectral change (Fig. 1), the P450d10m-ketoconazole complex is almost completely replaced by CO from the 6th ligand of the native sixth ligand (histidine) is stronger than that formed with the pyridine derivative, these imidazole and triazole derivatives can replace histidine from its binding place, i.e. the 6th co-ordination position of the haem iron. It has been suggested that nitrogen heterocycles with large hydrophobic N-1 substituents not only bind to the haem iron but also interact with the apoprotein [13]. Since both azole antifungals can be more easily replaced by CO from the 6th co-ordination position of P450d10m, it is tempting to speculate that the affinity of these azoles for the apoprotein of this

Table 1. Effects of azole antifungals on the formation of the reduced CO-P450 complexes.

<table>
<thead>
<tr>
<th>Species</th>
<th>Ketoconazole</th>
<th>Itraconazole</th>
<th>Fluconazole</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. cerevisiae</td>
<td>2.4</td>
<td>2.9</td>
<td>26.0</td>
</tr>
<tr>
<td>C. albicans</td>
<td>3.1</td>
<td>3.0</td>
<td>24.8</td>
</tr>
<tr>
<td>ATCC 28516</td>
<td>3.7</td>
<td>3.7</td>
<td>16.0</td>
</tr>
<tr>
<td>B41628</td>
<td>5.1</td>
<td>8.5</td>
<td>24.8</td>
</tr>
<tr>
<td>B44548</td>
<td>6.8</td>
<td>3.7</td>
<td>21.0</td>
</tr>
<tr>
<td>B44548 C–148</td>
<td>6.1</td>
<td>3.7</td>
<td>19.0</td>
</tr>
<tr>
<td>B44548 K–148</td>
<td>11.0</td>
<td>5.8</td>
<td>27.0</td>
</tr>
</tbody>
</table>

Fig. 1. Data taken from difference spectra obtained with microsomal preparations of S. cerevisiae SG1 (B53083, ATCC 46786, α-his 1–1, erg 11–1) (—–—) or its parent, S. cerevisiae DS87 (B53084) (—)
P450 is much lower than that for the apoprotein of the parent and other azole-sensitive fungi. This is in accord with the idea of Aoyama et al. [21] that the conformation of the apoprotein of P450_{so} is significantly altered in the substrate-binding site and/or haem environments.

The results discussed here suggest that the antifungal activity of the azole derivatives depends on the sensitivity of P450_{so}

Since the introduction of azole antifungals in 1971 only four cases of 'azole resistance' in *C. albicans* have been reported.

*C. albicans* isolate SH (B41628) was obtained from a patient with chronic mucocutaneous candidiasis, who relapsed after prolonged treatment with ketoconazole [22]. This isolate was less pathogenic than other *C. albicans* isolates in a number of animal models of infection [22]. For example, the *C. albicans* isolate B41628 might be regarded as non-pathogenic in the vaginal candidosis model in rats [22]. This might suggest that this *C. albicans* isolate is able to grow in immunocompromised patients only.

Investigations are in progress in an attempt to establish the mechanism of resistance of this *C. albicans* isolate to azole antifungals. Spectrophotometric studies show that the reduced CO-P450 complex(es) from the microsomal fraction of the ketoconazole-resistant *C. albicans* isolate, C44548 has maximum absorption at 450 nm instead of 448 nm (maximum absorption found with other *C. albicans* isolates). This P450_{so} shows low affinity for ketoconazole and fluconazole, but still relatively high affinity for itraconazole, and might suggest that the apoprotein of this isolate is altered in a region affecting the displacement seen withazole-sensitive *C. albicans* isolates [12, 21] and *S. cerevisiae* (Fig. 1). Much more stable ketoconazole-P450 complexes were obtained with microsomal suspensions of both *C. albicans* C-148 and K-148 (Fig. 2). However, the ketoconazole-P450 complex obtained with microsomes from *C. albicans* K-148 was more easily replaced by a CO-P450 complex (Fig. 2). The results obtained with K-148 resemble in some way those obtained with P450_{so} (Fig. 1) and might suggest that the apoprotein of this isolate is also altered in a region affecting the conformation of the haem environment.

The less stable complex formed with ketoconazole and the microsomal P450_{so} of *C. albicans* K-148 corresponds very well with its low effect on sterol synthesis by intact cells of the latter isolate (before inoculation in the test media cells were grown 24 h in drug-free CYG-medium). An IC_{50} value of 2.2 x 10^{-7} M was obtained with K-148, whereas for ergosterol synthesis by C-148 the IC_{50} was 9.2 x 10^{-5} M-ketoconazole. As could be expected from the affinity studies, itraconazole is a much more potent inhibitor of ergosterol synthesis in both K-148 and C-148. IC_{50} values of 3.2 x 10^{-7} M and 1.75 x 10^{-8} M are found, respectively.

Itraconazole also has a greater effect on the growth of *C. albicans* B44548 K-148 and C-148. IC_{50} values were 9 x 10^{-7} M and 4.1 x 10^{-7} M, respectively. With ketoconazole, IC_{50} values of 3.6 x 10^{-5} M (K-148) and 1.6 x 10^{-7} M (C-148) were found. Fluconazole had no effect on B44548 K-148, 36% inhibition of growth was reached at 10^{-4} M with C-148.

Further studies are in progress to establish the exact mechanism of resistance of this *C. albicans* isolate. These studies may be of help to prove further the hypothesis that binding of the hydrophobic N-1 substituent of the azoles to the apoprotein of the P450_{so} plays a key role in their antifungal activity. It may also improve our knowledge of P450_{so}, one of the most important targets for present antifungals of use in medicine as well as in plant protection.

We are grateful to Professor Y. Yoshida for providing us with *S. cerevisiae* strains SG, and D587, and to Dr. D. Warnock for the *C. albicans* isolates B41628 and B44548.

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**Fig. 2.** Data taken from difference spectra obtained with microsomal preparations of *C. albicans* B44548 C-148 (——) or *C. albicans* B44548 K-148 (---).

Further details are given in the legend to Fig. 1. The differences in absorption at 448.5 nm (B44548 C-148) or 449.5 nm (B44548 K-148) and 490 nm are plotted against time. ⊕, DMSO; O, itraconazole; ●, ketoconazole.
Inhibition of the sterol Δ⁴-reductase and Δ⁸ Δ⁷-isomerase in fungi

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Introduction
Several groups of chemically different compounds have shown to interfere with specific sites in sterol biosynthesis of plant and human pathogenic fungi [1-3]. Besides C-14 demethylation and squalene epoxidation, two other important sites in sterol biosynthesis have now been identified as targets for fungicides, namely the Δ⁴-reductase and the Δ⁸ → Δ⁷-isomerase.

Groups of chemicals inhibiting the Δ⁴-reductase and Δ⁸ → Δ⁷-isomerase comprise the cyclic amines [4-6], aza-steroids [3] and the azadecalins [7]. The cyclic amines are represented by the morpholines and piperidines. Of the morpholines, tridemorph, fenpropimorph and amorolfine are the most important representatives, whereas of the azadecalins 17). The cyclic amines are shown to interfere with specific sites in sterol biosynthesis of the membrane composition. As a consequence an irregular deposition of chitin is observed. Based on this observation differences in effects on the morphology of budding and filamentous fungi can be explained as well as the preferential inhibition of the yeast/hyphal transition in dimorphic fungi. Another consequence of membrane alterations is the impairment of primary metabolism. All these distortions lead to stasis of cell growth or the ultimate death of cells [1, 10].

Several cyclic amines and azadecalins have been shown to inhibit Δ⁴-reductase as well as Δ⁸ → Δ⁷-isomerase. The phenomenon of one compound acting at two different sites can only be explained if the mechanisms involved at these two sites have steps in common. Mechanistic studies indicate that similar carbocyclic high-energy intermediates are involved in the enzyme reactions of the Δ⁴-reductase and Δ⁸ → Δ⁷-isomerase [11]. After having given the state of the art of the research on inhibitors of Δ⁴-reductase and Δ⁸ → Δ⁷-isomerase of fungi, attention will be focused on factors influencing the antifungal activity and on confirmation of the high-energy intermediate hypothesis.

Factors influencing antifungal activity
Chemistry. The antiminotcic and agrifungicidal activities of cyclic amines are bound to specific structural requirements. Moreover, the stereochemistry proved to be very important when considering the differences in activity encountered during studies of the antifungal activity of the various iso- mers [4, 5]. Using the enzyme assays for Δ⁴-reductase and Δ⁸ → Δ⁷-isomerase in vitro, it has been proven that the differences in the effect of the various isomers on the growth of various fungi are reflections of the extent of the inhibition of the most sensitive enzyme to the most potent isomer [1, 10].

The antibiotic complex A25822B consists of seven structurally similar azasterols. Component A25822B (15-aza-methylene-D-homocholesta-8,14-dien-3β-ol) proved to be the most important compound in quantity as well as in activity. The similarity of the inhibitor to 8-sterol is evident.

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