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# INTERACTIONS OF IMIDAZOLE ANTIFUNGAL AGENTS WITH PURIFIED CYTOCHROME P-450 PROTEINS

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**Abstract**—The imidazole *N*-substituted antifungal agents ketoconazole, miconazole and clotrimazole have been shown to be potent inhibitors of oxidative metabolism by both a phenobarbital-induced cytochrome P-450 (P-450$_b$) and a 3-methylcholanthrene-induced cytochrome P-448-protein (P-450$_c$) in reconstituted systems. All three compounds inhibited the cytochrome P-450$_b$-dependent 7-pentoxyresorufin-*O*-dealkylase and the cytochrome P-450$_c$-dependent 7-ethoxyresorufin-*O*-deethylase activities. When 7-benzyloxyresorufin and 7-ethoxycoumarin were employed as substrates with both cytochrome preparations, all three antifungal compounds exhibited selective inhibition of the cytochrome P-450$_b$ preparation; ketoconazole was always the weakest inhibitor. The three antifungal agents were also shown to elicit a type II difference spectral interaction with both isoenzymes, the magnitude of the spectral interaction being greater with the cytochrome P-450$_b$ preparation.

Ketoconazole, and its analogues clotrimazole and miconazole, are well established drugs for the treatment of topical and systemic mycoses [1]. The fungistatic action of these *N*-substituted imidazoles is due to their potent inhibition of the microsomal cytochrome P-450-dependent 14$\alpha$-demethylation of lanosterol in ergosterol biosynthesis, which leads to loss of membrane integrity and subsequent inhibition of cell growth [2, 3]. Inhibition by these compounds is not limited to the cytochromes of fungi, and also occurs with a number of mammalian cytochromes P-450 including cytochrome P-450-dependent steroid metabolism in gonadal [4], placental [5], and adrenal tissues [6], as well as the hepatic microsomal oxygenation of a variety of xenobiotic compounds [7–10].

The isoenzymic characteristics of cytochrome P-450 are believed to arise from a "super family" of genes [11], and in the metabolism of xenobiotic compounds the two most extensively studied forms are those belonging to the two main gene families, namely, the phenobarbital-inducible cytochromes P-450 (cytochromes P-450$_b$ and P-450$_e$ of rat liver) and the polyaromatic hydrocarbon-inducible cytochromes P-448 (cytochromes P-450$_c$ and P-450$_d$). Following their successful solubilization and purification, it has been established that these two families of the haemoprotein differ in their structure and substrate specificities [12]. They possess distinctly different binding sites and often play contrasting roles in the bioactivation of drugs and chemical carcinogens [13, 14]. Computergraphic techniques have revealed that cytochromes P-448 interact essentially with long, planar molecules but not with bulky, nonplanar, molecules, whereas PB-cytochromes P-450 interact with globular molecules [15].

The aim of the present study was (a) to evaluate the inhibitory effects of ketoconazole, miconazole and clotrimazole in *in vitro* reconstituted systems employing purified cytochrome P-450$_b$ and cytochrome P-450$_c$ proteins, and (b) to establish if these inhibitors exhibit selectivity towards cytochromes P-450$_b$, as previously demonstrated in microsomal preparations [10].

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## MATERIALS AND METHODS

*Chemicals.* Clotrimazole (Sigma Co., Poole, Dorset, U.K.), 7-ethoxyresorufin, 7-pentoxyresorufin, 7-benzyloxyresorufin and resorufin (Molecular Probes, Junction City, OR) were all purchased. Miconazole and ketoconazole were generous gifts from Janssen Pharmaceutica (Wantage, Oxon, U.K.), as was benzphetamine HCl (Upjohn Co. Limited, 27100 le Vaudreil Ville, Naivelle, France) and Emulgen 911 (KAO Atlas Co, Tokyo, Japan). All other reagents and column chromatography media were purchased at the best available grade.

*Purification of the cytochromes and NADPH-cytochrome P-450 reductase.* Cytochrome P-450$_c$, cytochrome P-450$_b$ and NADPH-cytochrome P-450 reductase were purified from the hepatic microsomes of male Wistar albino rats (200–250 g). Rats from the Animal Breeding Unit, University of Surrey, were pretreated with either 3-methylcholanthrene, single daily intraperitoneal administrations (20 mg/kg) for three days, or phenobarbital (0.1% w/v in the drinking water for 10 days). All animals were killed 24 hr after the last administration of the inducing agent. The preparation and solubilization of microsomes have been previously described [16].

Cytochrome P-450$_c$ was purified by the method of Guengerich and Martin [17], as modified by Tamburini *et al.* [16]. NADPH-cytochrome P-450 reductase was purified from the microsomes of phenobarbital-pretreated animals as described by Yasukochi and Masters [18]. Cytochrome P-450$_b$ was eluted from DEAE-Sephacel during the reductase purification and was further purified by chroma-

tography on hydroxylapatite and CM-Sepharose CL-6B [19]. Emulgen 911 was removed from all three preparations using hydroxylapatite chromatography [16].

*Characterisation of the purified proteins.* The NADPH-cytochrome P-450 reductase preparation was electrophoretically homogeneous when loaded onto a sodium-dodecylsulphate–polyacrylamide gel, with a monomeric molecular weight of 76.2 Kd. The final specific content was 13.7 nmol/mg of protein, as determined by the method of French and Coon [20], exhibiting a specific activity of 44 units/mg (1 unit is equivalent to 1 μmol of cytochrome *c* reduced/min).

Cytochrome P-450$_c$ was also purified to electrophoretic homogeneity (>90% purity), having a monomeric molecular weight of 57.0 Kd, with a final specific content of 10.4 nmol cytochrome/mg protein [21]. The cytochrome P-450$_b$ protein was 80% pure on a protein basis (specific content 8.5 nmol/mg), the major band present exhibiting a molecular weight of 53.7 Kd; two contaminants were present at 48 Kd and 40 Kd, constituting 9% and 11% of the preparation, respectively, but it is highly unlikely that they represent phenobarbital-induced haemoproteins. Both cytochrome preparations exhibited a 415 nm/279 nm absorption ratio of 0.5 in the ferric state, indicating the presence of free apoprotein, and there was no detectable cytochrome P-420 in either preparation. Both proteins were predominantly low-spin haemoproteins, as determined by a wavelength maximum at 415 nm in the oxidized state.

The concentration of each cytochrome used in the reconstituted systems was based on the concentration of carbon monoxide binding protein, as determined by the method of Omura and Sato [21]. Both preparations were competent at metabolising the following substrates: benzphetamine (1 mM), 7-ethoxycoumarin (50 μM), 7-ethoxyresorufin (1 μM), 7-pentoxyresorufin (5 μM) and 7-benzyloxyresorufin (5 μM). Cytochrome P-450$_c$ exhibited the following turnovers: benzphetamine, 33; ethoxycoumarin, 15; ethoxyresorufin, 7.6; pentoxyresorufin, 0.06 and benzyloxyresorufin, 3.3 nmol/min per mol P-450$_c$ respectively, whereas for cytochrome P-450$_b$ these were: benzphetamine, 127; ethoxycoumarin, 3.0; ethoxyresorufin, 0.3; pentoxyresorufin, 0.4 and benzyloxyresorufin, 3.2 nmol/min per nmol P-450$_b$ respectively.

In addition, both cytochrome preparations were shown to be immunologically distinct as determined by a non-competitive ELISA method [22]. The preparation of antisera to cytochromes P-450$_c$ and P-450$_b$ was based on the method of Tamburini *et al.* [16]. The cytochrome P-450$_c$ preparation used in this study is believed to be similar to "cytochrome P-450$_c$" described by Ryan [12]. The cytochrome P-450$_b$ preparation is representative of the phenobarbital-inducible cytochromes P-450 and has similar characteristics to preparations previously purified in our laboratory [16]; catalytically, immunologically, spectrally and in terms of monomeric molecular weight, the preparation is similar to that of cytochrome "P-450 PB$_{3a}$" of Wolf *et al.* [23] and "cytochrome P-450$_b$" of Ryan [12].

*Reconstitution experiments.* The purified haemoproteins were reconstituted into previously sonicated L-α-dilauroyl phosphatidylcholine (15–30 μg/ml), in the presence of saturating amounts of the reductase (0.06–6.0 μM), according to the method of Haugen *et al.* [24]. The final concentration of the haemoprotein varied depending on the substrate employed (0.014–0.3 μM). All reactions were carried out at 37° in 50 mM HEPES buffer, pH 7.6, containing 0.1 mM EDTA and 15 mM $MgCl_2$. The reactions wre initiated with saturating amounts of NADPH, in 1% $NaHCO_3$, after a pre-incubation for 2 min at 37°. Inhibition studies were carried out by adding clotrimazole, miconazole and ketoconazole, dissolved in dimethylsulphoxide (DMSO 0.4% v/v), to the complete system and incubating for 3–4 min prior to the addition of NADPH. For the inhibition studies the concentration of the antifungal agents ranged from $10^{-8}$ to $10^{-6}$ M.

*Spectral studies.* The haemoproteins were suspended in 0.1 M potassium phosphate buffer, pH 7.4, containing 20% (v/v) glycerol, the final concentration of haemoprotein being 0.20 μM. The antifungal drugs were dissolved in dimethylformamide and 2 μl aliquots added to the sample cuvette, the same volume of solvent being added to the reference cuvette; binding spectra were recorded by the method of Schenkman [25], under oxidized conditions. Spectra were recorded using a Kontron-Uvikon 860 UV/visible spectrophotometer.

*Analytical procedures.* Protein content was determined by the method of Lowry *et al.* [26], as modified by Peterson [27], using bovine serum albumin as standard. Sodium-dodecylsulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out by the method of Laemmli [28], at a loading of 1 μg protein and the percentage purity of the purified cytochrome preparations was determined as described by Fenner *et al.* [29]. All gels were run at an acrylamide concentration of 10% (w/v). NADPH-cytochrome *c*-reductase and benzphetamine *N*-demethylase activities were determined according to the methods of Gibson and Schenkman [30] and Tamburini *et al.* [16] respectively. The *O*-dealkylations of 7-ethoxycoumarin, 7-ethoxyresorufin, 7-pentoxyresorufin and 7-benzyloxyresorufin were determined by direct fluorometric methods [23, 31]. The substrate concentrations employed in all assays were those previously shown to clearly distinguish between the phenobarbital- and 3-methylcholanthrene-induced proteins in microsomal preparations [10].

## RESULTS

The structures of the antimycotic agents employed in the present study are shown in Fig. 1. The addition of each of these compounds to cytochrome P-450$_c$ and cytochrome P-450$_b$ preparations yielded type II difference spectra. Binding to cytochrome P-450$_b$ was characterised by maxima and minima wavelengths of 429 nm and 391–396 nm, respectively, for clotrimazole and miconazole and 426 nm and 390–403 nm for ketoconazole (Fig. 2). Similar spectral interactions were observed with cytochrome P-450$_c$, the maximum being at 430 nm for all three

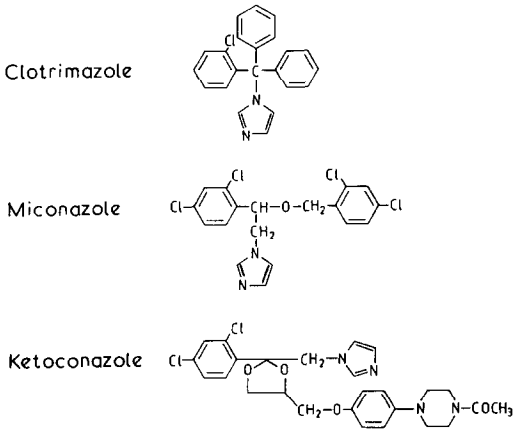


Fig. 1. Chemical structures of clotrimazole, miconazole and ketoconazole.

compounds, while wavelength minima ranged from 408 nm for clotrimazole to 400 nm for miconazole. The magnitude of the spectral interactions was found to be greater with the cytochrome P-450$_b$ than with the cytochrome P-450$_c$ in all three cases, the greatest difference being observed for miconazole (Table 1).

All three compounds were shown to be inhibitors of both the cytochrome P-450$_b$- and the cytochrome P-450$_c$-dependent activities, as exemplified by their effect on the metabolism of the marker substrates 7-pentoxyresorufin and 7-ethoxyresorufin, respectively (Table 2). With both purified proteins, ketoconazole was the weakest inhibitor while miconazole and clotrimazole appeared to be equipotent. All three imidazoles were more potent inhibitors of the cytochrome P-450$_b$-catalysed 7-pentoxyresorufin dealkylase than the cytochrome P-450$_c$-catalysed 7-ethoxyresorufin *O*-deethylase activity.

When 7-ethoxycoumarin and 7-benzyloxyresorufin were employed as substrates, for both purified protein preparations, all three antimycotics exhibited some degree of selectivity for the cytochrome P-450 preparation (Tables 3 and 4). Once again ketoconazole was the weakest inhibitor.

Table 1. Spectral interactions of ketoconazole, miconazole and clotrimazole with purified cytochrome P-450-proteins

| | $\Delta A_{max}$ (nmol P-450)$^{-1}$ | |
|---|---|---|
| Compound | Cytochrome P-450$_c$ | Cytochrome P-450$_b$ |
| Clotrimazole | 0.035 | 0.045 |
| Miconazole | 0.015 | 0.055 |
| Ketoconazole | 0.030 | 0.035 |

The ligand (12 $\mu$M for each drug) was dissolved in dimethylformamide and added to the test cuvette which also contained the purified protein (0.2 $\mu$M). Reference cuvette contained only the protein and the corresponding volume of the solvent. $A_{max}$ is defined as the difference between peak and trough absorbances, at the saturating concentration of ligand employed.

Miconazole and ketoconazole did not inhibit the NADPH-cytochrome *c*-reductase activity, as mediated by the purified flavoprotein, at the concentrations of inhibitor employed ($10^{-8}$ M–$10^{-4}$ M). Clotrimazole, however, caused a 47% inhibition only at the highest concentration of $10^{-4}$ M (results not shown).

## DISCUSSION

The observation that clotrimazole, miconazole and ketoconazole were potent inhibitors of cytochrome P-450-dependent oxidative metabolism is in agreement with our previous findings and those of others using microsomal preparations [8–10, 32, 33]. The purpose of the present study was to ascertain if these agents show selectivity towards phenobarbital-induced cytochrome P-450$_b$ or polyaromatic hydrocarbon-induced cytochrome P-450$_c$ by employing reconstituted systems containing the respective purified enzyme proteins. 7-Ethoxyresorufin and 7-pentoxyresorufin are now well established, highly specific marker substrates for cytochromes P-450$_c$ and cytochromes P-450$_b$ respectively [23, 31, 34], and have been used in this study to monitor the activity of these haemoproteins. Both miconazole and clo-

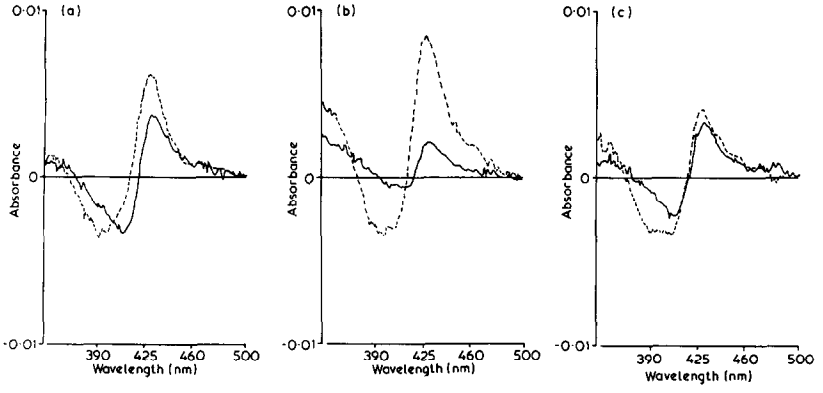


Fig. 2. Interaction of imidazole antifungals with purified cytochrome P-450$_b$ and cytochrome P-450$_c$. (——), Cytochrome P-450$_c$ preparation was placed in both reference and sample cuvettes while (a) clotrimazole, (b) miconazole and (c) ketoconazole was added to sample cuvette only. (- - - -), The experiment was repeated with the cytochrome P450$_b$ preparation. The final concentation of ligand and haemoprotein were 12 $\mu$M and 0.2 $\mu$M, respectively. For conditions see Materials and Methods.

Table 2. Inhibition of 7-ethoxyresorufin and 7-pentoxyresorufin metabolism by antifungal agents in reconstituted systems

| | Cytochrome P-450 activity (pmol/min/nmol cyt. P-450) | | | |
|---|---|---|---|---|
| | Ethoxyresorufin activity of cytochrome P-450$_c$ | | Pentoxyresorufin activity of cytochrome P-450$_b$ | |
| | 1 | 2 | 1 | 2 |
| Control | 5310 | 6910 | 210 | 240 |
| Ketoconazole | 4860 (92) | 7100 (103) | 128 (61) | 90 (38) |
| Miconazole | 3599 (66) | 3830 (55) | 30 (14) | 23 (10) |
| Clotrimazole | 3500 (66) | 4260 (62) | 34 (16) | 23 (10) |

Two separate experiments were carried out in the presence of inhibitors, at a concentration of $1.3 \times 10^{-7}$ M dissolved in DMSO. Control experiments were carried out in the presence of 0.4% (v/v) DMSO only. The values in parentheses are % activities remaining relative to control.

trimazole, and to a lesser extent ketoconazole, were shown to exhibit some degree of selectivity towards the cytochrome P-450$_b$ preparation.

When the inhibition of 7-ethoxycoumarin and 7-benzyloxyresorufin metabolism, in both haemoprotein preparations, was considered all three compounds were shown to be selective towards cytochrome P-450$_b$ (Tables 3 and 4). 7-Ethoxycoumarin, for example, exhibited similar $K_m$ values for both purified preparations, namely, $8.3 \pm 2.4$ μM and $13.6 \pm 2.5$ μM for cytochrome P-450$_c$ and cytochrome P-450$_b$ respectively (data not shown). Since the substrate affinities of both cytochromes for 7-ethoxycoumarin are similar, the difference in inhibitory potency is attributable to differences in the interactions of the inhibitors with the haem moiety or to competition by the inhibitor for the substrate binding sites of the two cytochromes. We have recently reported similar findings using hepatic microsomal preparations from 3-methylcholanthrene and phenobarbital-treated rats [10]. The finding that clotrimazole is selective for the PB-induced protein is in agreement with the findings of Kahl *et al.* [8]. In studies *in vivo*, however, ketoconazole has been reported to be a more specific inhibitor of the cytochrome P-450$_c$-dependent caffeine *N*-demethylation than the cytochrome P-450$_b$-dependent aminopyrine *N*-demethylation [7].

With both preparations and with all substrates, ketoconazole was always the weakest inhibitor which lends support to other studies employing different substrates [5, 32, 35, 36]. It is now well documented that, in general, 1′-substituted imidazoles inhibit oxidative metabolism by primarily ligating directly, and reversibly, to the sixth coordination site of the haem [37]. None of the compounds inhibited NADPH-cytochrome *c* reductase activity at the concentrations where inhibition of oxidative metabolism was evident, showing that these potent inhibitors do not interfere with the electron flow to the terminal haemoprotein. Similar observations have been made with hepatic microsomal preparations [5, 9]. Furthermore, these compounds give rise to typical type II difference spectral interactions and compete with carbon monoxide for ferrous cytochrome P-450 [8, 32, 38]. In the present study, all three compounds elicited type II difference spectra with both purified proteins. The magnitude of the spectral interactions, in the presence of saturating concentrations of the ligand (12 μM), were markedly greater for cytochrome P-450$_b$ with miconazole and to a lesser degree with clotrimazole and ketoconazole (see Table 1). However, the values obtained in Table 1 can only be

Table 3. Inhibition of 7-ethoxycoumarin-*O*-deethylase activity by antifungal agents in reconstituted systems

| | Cytochrome P-450 activity (pmol/min/nmol cyt. P-450) | | | |
|---|---|---|---|---|
| | Cytochrome P-450$_c$ | | Cytochrome P-450$_b$ | |
| | 1 | 2 | 1 | 2 |
| Control | 10,500 | 7350 | 4330 | 2540 |
| Ketoconazole | 10,100 (96) | 6120 (83) | 1530 (36) | 1160 (46) |
| Miconazole | 8400 (80) | 4900 (67) | 1220 (28) | 700 (28) |
| Clotrimazole | 7600 (72) | 4490 (61) | 1070 (25) | 760 (30) |

Two separate experiments were carried out in the presence of inhibitors, at a concentration of $1.3 \times 10^{-7}$ M dissolved in DMSO. Control experiments were carried out in the presence of DMSO only. The values in parentheses are % activities remaining relative to control.

Table 4. Inhibition of 7-benzyloxyresorufin-*O*-debenzylase activity by antifungal agents in reconstituted systems

| | $I_{50}$ (M) | |
|---|---|---|
| Compound | Cytochrome P450$_c$ | Cytochrome P-450$_b$ |
| Clotrimazole | $9.0 \times 10^{-8}$ | $5.0 \times 10^{-8}$ |
| Miconazole | $1.5 \times 10^{-7}$ | $2.8 \times 10^{-8}$ |
| Ketoconazole | $>1 \times 10^{-6}$ (68%) | $7.1 \times 10^{-7}$ |

Control activities were $3.2 \pm 0.5$ and $1.4 \pm 0.5$ nmol/min per nmol of haemoprotein for cytochrome P-450 and cytochrome P-450$_b$ respectively. Value in parentheses reflects remaining enzyme activity at an inhibitor concentration of $1 \times 10^{-6}$ M.

considered as estimates of the extent of binding ($\Delta A_{max}$). There is normally a good correlation between the spectrally determined constant $\Delta A_{max}$ and kinetically determined $I_{50}$ values [32], although the experimental conditions employed in these studies are markedly different.

Although the direct ligation to the haem is recognised as the primary mechanism of inhibition, there is considerable evidence to suggest that an additional hydrophobic interaction, between the non-imidazole moiety and the substrate-binding site, is involved [10, 38, 39]. Ketoconazole and econazole undergo oxidative metabolism *in vivo* indicating that they are mixed-function oxidase substrates [40, 41]. Furthermore, 1'-substituted imidazoles exhibit complex inhibition kinetics and have been shown to displace type I ligands from the substrate binding site of cytochrome P-450 [10, 32, 42]. Finally, 1-(4-azidophenyl)-imidazole has been successfully used as a photo-affinity probe for studying the type I active site of cytochrome $P\text{-}450_{cam}$ [43].

The selectivity of the antifungal agents for cytochrome $P\text{-}450_b$ has been predicted with the use of computergraphic techniques [10]. Molecules that interact with cytochromes $P\text{-}450_c$ are essentially planar, characterized by a large area/depth ratio and a small depth, whereas cytochrome $P\text{-}450_b$ can accommodate bulky, non-planar molecules having a small area/depth ratio and a larger depth [15]. All three antifungal agents clearly fall into the second category and indeed are more potent inhibitors of the cytochrome $P\text{-}450_b$-dependent enzyme activities as shown in the present study with purified haemoproteins and previously with microsomal preparations [10].

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