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# INHIBITION OF RAT LIVER MICROSOMAL CYTOCHROME P-450 STEROID HYDROXYLASE REACTIONS BY IMIDAZOLE ANTIMYCOTIC AGENTS*

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**Abstract**—The imidazole antimycotic agents ketoconazole, miconazole and clotrimazole were tested for their abilities to inhibit the reactions involved in the oxidative metabolism of androst-4-ene-3,17-dione by rat liver microsomal cytochromes P-450. All three compounds were found to function as potent inhibitors of steroid hydroxylase reactions, producing 50% inhibition of 6$\beta$-, 16$\beta$-, and 16$\alpha$-hydroxylase activities at concentrations between $10^{-7}$ and $10^{-5}$ M. The antimycotic agents, when added to liver microsomes, bound to cytochrome P-450 with high affinity to produce a "type II" spectral complex. These agents showed differential inhibition of the various steroid hydroxylases and were found not to affect the activities of the liver microsomal steroid 5$\alpha$-reductase or the androst-4-ene-3,17-dione 17-oxidoreductase. The results presented demonstrate an interaction of these imidazole antimycotic agents with the various cytochromes P-450 of liver microsomes, resulting in selective inhibition of monooxygenase activity.

Liver microsomal cytochromes P-450 consist of a family of closely related enzymes which are unique in their abilities to metabolize a variety of lipophilic foreign and endogenous compounds. It is generally recognized that steroid hormones may constitute physiological substrates of liver microsomal cytochromes P-450 [1, 2]. These enzymes metabolize steroid hormones by oxidatively substituting a hydroxyl group at specific sites on the steroid nucleus to produce metabolites that are more polar. The hydroxylated steroid metabolites are thereby made more accessible for subsequent conjugation reactions which may facilitate further their excretion in the urine and bile.

Ketoconazole, miconazole and clotrimazole are three imidazole antimycotic agents which have been described recently to retard fungal growth [3]. Their mechanism of action is to inhibit the cytochrome P-450-dependent 14$\alpha$-demethylase activity required for the conversion of lanosterol to ergosterol in yeast [3, 4]. The structures of these three antimycotic agents are shown in Fig. 1. Ketoconazole, miconazole and clotrimazole have been found to inhibit a number of other cytochrome P-450-dependent enzyme activities of gonadal, placental and adrenal tissues which function in steroid hormone biosynthesis [5–9]. In the liver and epidermis, clotrimazole has been shown to prevent the formation of benzo[*a*]-pyrene metabolite-DNA adducts *in vitro* as catalyzed by microsomal preparations from phenobarbital- or 3-methylcholanthrene-treated rats [10, 11]. In addition, we have shown recently that ketoconazole acts as a potent inhibitor of drug monooxygenase activities as catalyzed by rat liver microsomal cytochromes P-450 [12].

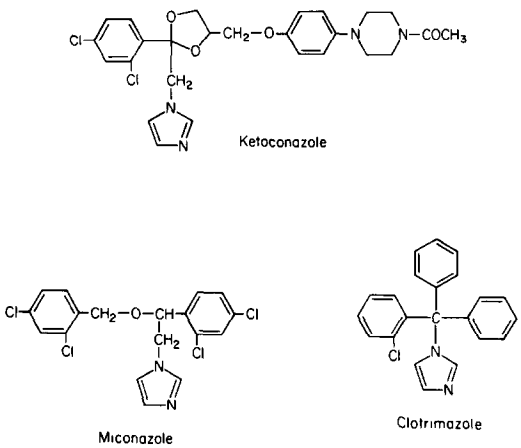


Fig. 1. Chemical structures of ketoconazole, miconazole, and clotrimazole.

The ability of liver microsomal cytochrome P-450 isozymes to catalyze site-specific steroid hydroxylation reactions [13, 14] allows the opportunity to monitor simultaneously the activities of a variety of cytochromes P-450 during the metabolism of a single substrate. A quantification of the hydroxylated

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metabolites, in the presence of various concentrations of the antimycotic agents, should allow us to determine differences in individual dose responses of the cytochrome P-450 isozymes of liver microsomes which metabolize steroids. Therefore, we have determined the potencies of three imidazole antimycotic agents to inhibit the various microsomal cytochrome P-450 reactions involved in the metabolism of androstenedione*.

## MATERIALS AND METHODS

*Chemicals.* Miconazole nitrate and clotrimazole were obtained from the Sigma Chemical Co. (St. Louis, MO). Ketoconazole was supplied by Janssen Life Products, Inc. (Piscataway, NJ). [4-$^{14}$C]-Androst-4-ene-3,17-dione (sp. act. 59 mCi/mmole) was from Amersham (Arlington Heights, IL). 6$\beta$-, 7$\alpha$-, and 16$\alpha$-Hydroxyandrost-4-ene-3,17-dione and androst-5-ene-3$\beta$,16$\beta$-diol-17-one were supplied by the MRC Steroid Reference Collection, London. 16$\beta$-Hydroxyandrost-4-ene-3,17-dione was synthesized by the procedure of Talalay and Dobson [15], from androst-5-ene-3$\beta$,16$\beta$-diol-17-one using partially purified 3$\beta$-hydroxysteroid dehydrogenase from *Pseudomonas testosteroni* (Sigma Chemical Co.). Androst-4-ene-3,17-dione and 17$\beta$-hydroxyandrost-4-en-3-one were from Steraloids Inc. (Wilton, NH). All other chemicals were of the highest purity available from commercial supply houses.

*Preparation of microsomes.* Male Sprague–Dawley rats (150–200 g) were given a single intraperitoneal injection of phenobarbital (80 mg/kg, dissolved in water) followed by inclusion of phenobarbital (0.05%) in their drinking water for 4 days. Female rats received no drugs. The animals were allowed food and water *ad lib.*, but were starved overnight, prior to decapitation. Livers were perfused *in situ* with ice-cold sodium chloride (0.9%), pooled, and homogenized in 5 vol. of sucrose (0.25 M) using a Potter–Elvehjem Teflon pestle tissue homogenizer. The microsomal fraction was obtained as described [16]. Protein concentrations were determined by the Biuret method [17]. Cytochrome P-450 content was determined spectrophotometrically by the method of Omura and Sato [18]. The specific content of cytochrome P-450 contained in the liver microsomes ranged from 0.8 to 0.9 and 3.0 to 3.6 nmoles of cytochrome P-450 per mg protein for microsomes obtained from female and phenobarbital-pretreated male rats respectively.

*Metabolism of androstenedione.* Rat liver microsomes (0.25 mg protein/ml) were suspended in a reaction mixture consisting of 50 mM Tris–chloride buffer (pH 7.5), 150 mM KCl, 10 mM $MgCl_2$, 8 mM sodium isocitrate and 0.5 units/ml isocitrate dehydrogenase in a final volume of 2 ml. [4-$^{14}$C]-Androst-4-ene-3,17-dione (0.14 $\mu$Ci/ml) was added from a 20 mM solution to give a final concentration of 100 $\mu$M. Various concentrations of the antimycotic agents, dissolved in ethanol, were added and the reaction mixture was preincubated at 37° for 1 min. The reaction was initiated by the addition of NADPH to give a final concentration of 0.5 mM. After incubating for 5 min at 37°, the reaction was terminated by the addition of 5 ml of methylene chloride; the mixture was vortexed and placed on ice. The mixture was extracted three times with methylene chloride (5 ml) which removed 99% of the radioactivity from the aqueous phase. The organic extract was dried with $Na_2SO_4$, filtered through a 0.45 $\mu$m filter, and evaporated to dryness at 30° under a stream of nitrogen. The residue containing the steroid metabolites was dissolved in 100 $\mu$l of methanol and analyzed by reverse phase ($C_{18}$) HPLC using a linear gradient of 20–45% aqueous $CH_3CN$ for 40 min followed by isocratic chromatography at 45% $CH_3CN$ for 14 min at a flow rate of 1 ml/min. The elution of metabolites was monitored spectrophotometrically at 254 nm and by collecting fractions (0.3 ml) and quantification of radioactivity by scintillation counting.

Identification of the metabolites of androstenedione was made by comparison of their retention times with known standards on HPLC and GC and by their mass spectrum. In addition, the metabolites were acetylated by the procedure of Dominguez *et al.* [19], and their retention times on HPLC and GC were compared to those of acetylated authentic standards for further confirmation of identity. Multiple-hydroxylated metabolites of androstenedione accounted for less than 1.5% of the total amount of metabolites detected.

*Binding difference spectra of rat liver microsomal cytochrome P-450.* Liver microsomes from male rats pretreated with phenobarbital were suspended to a concentration of 0.67 mg protein/ml, and androstenedione (100 $\mu$M) was added. The mixture was divided equally between the sample and reference cuvettes. Clotrimazole, dissolved in ethanol, was added to the sample cuvette and an equal volume of ethanol was added to the reference cuvette. After a 10-min equilibration period, the resulting difference spectrum was recorded on an Aminco DW-2 split bean UV/VIS spectrophotometer. The final concentration of ethanol never exceeded 0.5%.

## RESULTS

Microsomes prepared from livers of male rats pretreated with phenobarbital were used to assay for steroid metabolism since phenobarbital pretreatment increased the rates of 6$\beta$- and 16$\beta$-hydroxylation as well as the overall rate of metabolism of androstenedione and other steroids [14, 20]. In addition, these preparations contain a relatively low level of microsomal androstenedione 5$\alpha$-reductase activity [20, 21]. To quantify steroid metabolism, androstenedione was used as the substrate since this steroid is oxidatively metabolized by rat liver microsomal cytochromes P-450 at a rapid rate. An HPLC system was optimized to separate all known metabolites of androstenedione and to detect the presence of as yet unidentified metabolites. Figure 2A presents a high performance liquid chromatogram of the metabolites extracted after an incubation of andro-

* Abbreviations: androstenedione, androst-4-ene-3,17-dione; 5$\alpha$-androstanedione, 5$\alpha$-androstane-3,17-dione; testosterone, 17$\beta$-hydroxyandrost-4-en-3-one; HPLC, high performance liquid chromatography; and GC, gas chromatography.

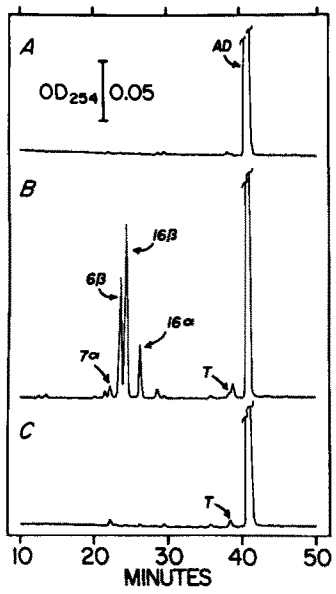


Fig. 2. High performance liquid chromatograms of the metabolites of androstenedione. Androstenedione (100 μM) was incubated for 5 min at 37° with liver microsomes (0.25 mg protein/ml) from rats pretreated with phenobarbital. The metabolites were extracted and analyzed as described in Materials and Methods. Key: (A) absence of NADPH; (B) presence of NADPH (0.5 mM); and (C) presence of NADPH (0.5 mM) plus clotrimazole (10 μM). The respective hydroxylated metabolites of androstenedione are identified by 7α, 6β, 16β and 16α. AD and T are androstenedione and testosterone respectively.

stenedione (100 μM) for 5 min in the absence of NADPH with liver microsomes from rats pretreated with phenobarbital. Under these conditions, only the presence of unreacted androstenedione can be detected by the absorptivity at 254 nm. This was also the only steroid present as detected by determination of radioactivity in collected fractions (data not shown). In the presence of NADPH, as shown in Fig. 2B, a number of metabolites are formed, including the 6β-, 7α-, 16α- and 16β-hydroxyandrostenediones, testosterone and other minor metabolites of androstenedione which have not yet been identified. A small amount of 5α-androstanedione, which cannot be detected by monitoring absorptivity at 254 nm, was also formed by male rat liver microsomes at a rate of 0.3 nmole/min/mg protein as determined by the radioactivity which eluted at 48.2 min. All of the other radiolabeled fractions corresponded to the metabolites having absorbance at 254 nm shown in Fig. 2B. Figure 2C shows that, in the presence of clotrimazole (10 μM), almost complete inhibition of the formation of all the steroid metabolites occurred, except for 7α-hydroxyandrostenedione which was inhibited only slightly more than 50% and for testosterone and 5α-androstanedione, the formation of which were not affected by the presence of this concentration of clotrimazole. Neither the 17-oxidoreductase which forms testosterone nor the 5α-reductase enzymes involve cytochromes P-450.

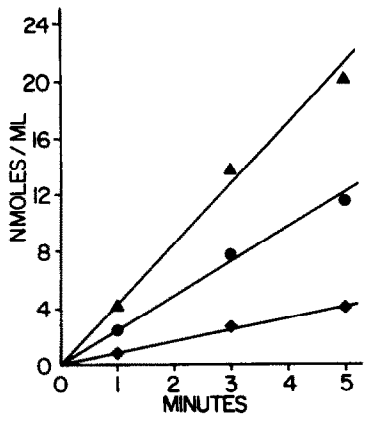


Fig. 3. Time course for the formation of 16β-, 6β- and 16α-hydroxyandrostenediones. Androstenedione (100 μM) was incubated with liver microsomes (0.25 mg protein/ml) for various times in the presence of NADPH (0.5 mM), and the metabolites were separated by HPLC and quantified by liquid scintillation counting. Key: (▲) 16β-hydroxyandrostenedione; (●) 6β-hydroxyandrostenedione, and (◆) 16α-hydroxyandrostenedione.

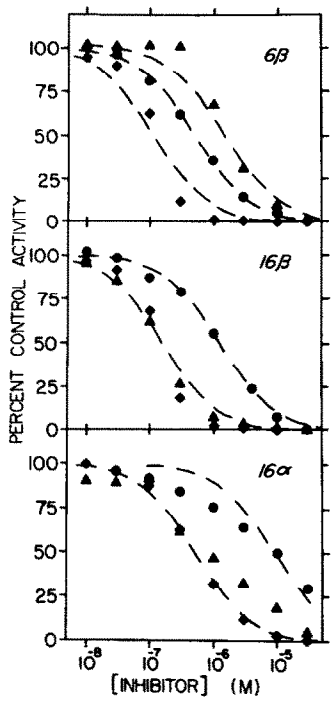


Fig. 4. Dose–response curves for the inhibition of the androstenedione 6β-, 16β- and 16α-hydroxylase activities by imidazole antimycotic agents. Androstenedione (100 μM) was incubated with rat liver microsomes for 5 min at 37° in the presence of various concentrations of miconazole (▲), clotrimazole (◆) or ketoconazole (●). Control rates in the absence of inhibitor averaged 9.1, 13.6 and 3.2 nmoles/min/mg protein for the 6β (top panel), 16β (middle panel) and 16α (bottom panel) hydroxylase activities respectively. Data points represent the mean of two separate experiments. Dashed curves represent theoretical dose responses for an inhibitor having a binding constant of 0.1, 0.5 and 2.0 μM for the 6β-hydroxylase, 0.12 and 1.0 μM for the 16β-hydroxylase, and 3.0 and 10.0 μM for the 16α-hydroxylase.

Figure 3 shows the time course for the production of the three most predominant metabolites formed by incubating androstenedione with liver microsomes from male rats pretreated with phenobarbital. The rates of formation of the 6β-, 16α- and 16β-hydroxyandrostenediones were linear for at least 5 min. The initial rates of formation of these three metabolites were also determined to be directly dependent on the microsomal protein concentration up to 0.5 mg/ml (data not shown).

To determine the inhibitory potencies of the various imidazole antimycotic agents on the different steroid hydroxylase reactions, a measure of activity versus inhibitor concentration was determined. Figure 4 shows the dose–response curves for the inhibition of 6β-, 16β- and 16α-hydroxylase activities by ketoconazole, miconazole and clotrimazole. The three antimycotic agents were found to act as potent inhibitors of the 16β-hydroxylase activity, producing 50% inhibition at concentrations between $10^{-7}$ and $10^{-6}$ M. In contrast, imidazole at a concentration of $10^{-4}$ M resulted in only 30% inhibition of the 16β-hydroxylase activity and negligible effect on the 6β- or 16α-hydroxylase activities (data not shown). Clotrimazole was determined to be the most potent inhibitor of the oxidative metabolism of androstenedione. This antimycotic agent was an equally potent inhibitor of both the 6β- and 16β-hydroxylation reactions ($I_{50}$ = 0.12 μM) but was less potent to inhibit 16α-hydroxylation. Miconazole was found to be equally effective at inhibiting 16β-hydroxylation as clotrimazole, but required greater than ten times higher concentration to inhibit 6β-hydroxylation. Ketoconazole required the highest concentration of the three compounds tested to inhibit 16α- and 16β-hydroxylation, but was more potent than miconazole in inhibiting 6β-hydroxylation. The imidazole antimycotic agents also inhibited the formation of 7α-hydroxyandrostenedione as well as the other unidentified hydroxylated metabolites of androstenedione. The low activity of these reactions as compared to the 6β-, 16β- and 16α-hydroxylase reactions prevented an accurate measurement of their response to the imidazole antimycotic agents.

Addition of the imidazole antimycotics to liver microsomes resulted in the formation of a type II binding difference spectrum. Figure 5 shows that clotrimazole bound both tightly and saturably to the cytochrome P-450 component of microsomes to form a ferrihemochrome complex. An accurate determination of the dissociation constant was difficult to measure due to near one-to-one stoichiometric binding of clotrimazole to the hemoprotein when the ligand was added at low concentrations.

Two other microsomal reactions which do not involve cytochrome P-450 enzymes also function to metabolize androstenedione in liver. These include a 17-oxidoreductase which converts androstenedione to testosterone, and a 5α-reductase which reduces the $\Delta^4$ double bond of androstenedione to form 5α-androstanedione. In the presence of a 30 μM concentration of the imidazole antimycotic agents, the 17-oxidoreductase activity decreased by only 5–15% (data not shown). In contrast, this concentration of the various antimycotic agents resulted in 70–100% inhibition of the microsomal steroid hydroxylase

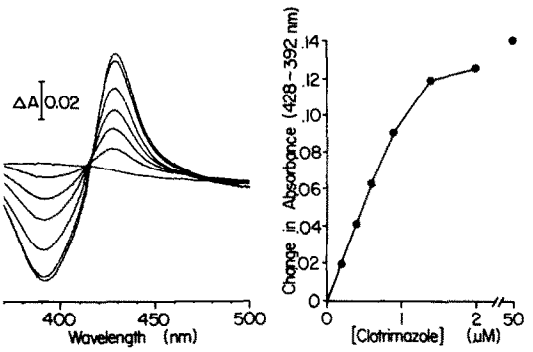


Fig. 5. Spectral titration of liver microsomal cytochrome P-450 with clotrimazole. Left panel: microsomes from male rats pretreated with phenobarbital were suspended to 0.67 mg protein/ml (2.0 μM P-450) in assay buffer. Androstenedione (100 μM) was added to both the sample and reference cuvettes, and difference spectra were recorded after successive additions of 0.2, 0.4, 0.6, 0.9, 1.4, 2.0 and 50.0 μM (not shown) clotrimazole to the sample cuvette. Right panel: plot of the spectral change versus concentration of clotrimazole.

activities, as shown in Fig. 4. Similarly, the antimycotic agents had little or no effect on the level of androstenedione 5α-reductase activity when added at concentrations between 10 and 100 μM (data not shown).

## DISCUSSION

The results presented in this study demonstrate that ketoconazole, miconazole, and clotrimazole are potent and specific inhibitors of the liver microsomal cytochrome P-450 reactions involved in the oxidative metabolism of androstenedione. These imidazole antimycotic agents produced 50% inhibition of the various steroid hydroxylase activities at concentrations between $10^{-7}$ and $10^{-5}$ M. In addition, these agents showed selectivity in their potency to inhibit the various steroid hydroxylase reactions. At least a 5-fold difference in concentration was required of the three antimycotic agents to inhibit the 6β-hydroxylase activity by 50%. Clotrimazole was determined to be the most potent inhibitor of steroid hormone hydroxylase activity. This compound inhibited the 6β-, 16β- and 16α-hydroxylase activities by 50% at concentrations of less than 1 μM.

The data points in Fig. 4 representing the androstenedione 16α-hydroxylase activity substantially deviated from a sigmoidal-shaped curve when plotted versus the logarithm of either miconazole or ketoconazole concentration. This result would have occurred if multiple microsomal cytochromes P-450, having substantially different affinities for the antimycotic agents, had participated in the 16α-hydroxylation of androstenedione. This interpretation is consistent with reports of multiple forms of purified rat liver microsomal cytochromes P-450 capable of catalyzing 16α-hydroxylation of testosterone or androstenedione in a reconstituted enzyme assay system [14, 22]. The sigmoidal shape of the dose–response curve for the inhibition of 16α-hydroxylase activity by clotrimazole, however, suggests that this

inhibitor may have a similar high affinity for the multiple 16α-hydroxylase enzymes. Studies of the binding of these imidazole antimycotic agents to purified forms of cytochromes P-450 will be required in order to measure the interaction of these compounds with each of the individual microsomal cytochromes P-450 which catalyze 16α-hydroxylation of androstenedione.

Previous studies have shown that the antimycotic agents do not affect the activity of NADPH-cytochrome *c* (P-450) reductase [7, 12] and thus do not impede the transfer of reducing equivalents to cytochrome P-450. The inhibition of the various steroid hydroxylase activities by the antimycotic agents is believed to result from a direct interaction of these compounds with cytochrome P-450. All three of the antimycotics used in this study bind with high affinity to liver microsomal cytochromes P-450 to produce a type II difference binding spectrum [10, 12]. This spectral change is believed to result from a coordination of an imidazole nitrogenous ligand to the heme-iron of cytochrome P-450 which results in the inhibition of enzymatic activity [23, 24].

The abilities of these three compounds to inhibit the monooxygenase activities involved in steroid hormone metabolism in liver microsomes, as well as a variety of monooxygenase activities involved in drug metabolism and steroid hormone biosynthesis [6–12], suggest that the imidazole group of these three antimycotic agents, when bound to the hemoprotein, is able to orientate itself in close proximity to the heme-iron of a variety of cytochromes P-450. The role of the remaining portion of the molecule to enhance binding, and possibly facilitate the coordination of the imidazole nitrogen atom to the hemoprotein, remains to be defined. The broad capacity and high potency of these agents to inhibit a variety of monooxygenase activities may prove valuable for their possible use as probes for determining whether a reaction involves cytochrome P-450.

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