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COMPARATIVE EFFECTS OF ANTITHROMBITIC AND ANTIMYCOTIC *N*-SUBSTITUTED IMIDAZOLES ON RAT HEPATIC MICROSOMAL STEROID AND XENOBIOTIC HYDROXYLASES *IN VITRO*

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Abstract—*N*-Substituted imidazoles have been shown to be potent inhibitors of microsomal mixed-function oxidase activities *in vitro* and *in vivo*. In the present study the effects of two antithrombitic (dazmegrel and dazoxiben) and four antimycotic (ketoconazole, econazole, miconazole and clotrimazole) imidazoles on microsomal cytochrome P-450-mediated steroid and xenobiotic hydroxylases were studied *in vitro*. Despite the presence of the *N*-substituted imidazole moiety, the antithrombitic agents were essentially non-potent as inhibitors of all of the oxidase activities evaluated. In contrast, the antimycotic drugs were potent inhibitory compounds. Binding studies revealed that all six imidazoles elicited type II optical difference spectra and exhibited relatively high affinity for ferricytochrome P-450 in microsomal suspensions (K_s range 0.26–0.73 μM for the antimycotic agents and 6.5 μM and 21 μM for dazmegrel and dazoxiben, respectively). The structural feature that the antithrombitic compounds share is a carboxylate function so that, at physiological pH, less than 1% of the drug would be present in the unionised form. This functionality is absent from the structures of the antimycotic agents which possess much greater hydrophobic character. Even though the antithrombitic imidazoles elicit type II binding interactions of quite high affinity it would appear from this study that significant inhibition potency does not necessarily follow. The present findings also suggest that interesting differences exist between the active site binding regions in the cytochrome P-450 that catalyse thomboxane synthetase activity and those involved in microsomal drug oxidation. Inhibitor hydrophobicity is clearly an important factor in the inhibition of microsomal cytochromes P-450 whereas effective thromboxane synthetase inhibitors may be quite hydrophilic at physiological pH.

Cytochromes P-450 (P-450) have important catalytic functions in the biosynthesis and oxidative degradation of many substances of physiological importance. Many lipophilic compounds, of exogenous and endogenous origin, are converted to more hydrophilic metabolites by the hepatic microsomal P-450 system. For example, the steroid hormones are considered to be the physiological substrates of the hepatic P-450s and recent studies have established that individual P-450s catalyze the regio- and stereospecific hydroxylation of androgens and oestrogens [1–4].

Several classes of drugs are inhibitors of P-450 systems involved in biosynthetic and bioinactivation pathways. Perhaps the most potent inhibitors are the imidazoles [5–7] and some derivatives, specifically the antithrombitic imidazoles, are employed in clinical situations that take advantage of their capacity to inhibit the platelet P-450-thromboxane synthetase [8, 9]. Other imidazoles may potentiate the pharmacological action of co-administered drugs as a result of non-specific inhibition of P-450-mediated catalysis. Many of the antimycotic imidazole drugs are potent inhibitors of P-450 systems involved in steroidogenesis and thromboxane B_2 biosynthesis [10–12]. However, little information is available regarding the effect of antithrombitic imidazoles on the hepatic P-450-mediated drug oxidases.

The present study was undertaken to determine the effects of two imidazoles with established antithrombitic activity on rat liver microsomal steroid hydroxylases. Comparative studies were also conducted with a series of four antimycotic imidazoles, since these agents are known to be effective inhibitors of the microsomal mixed-function oxidases. Finally, the capacity of the imidazole drugs to bind to ferric P-450 was estimated in microsomal fractions in relation to steroid hydroxylase inhibition data.

EXPERIMENTAL

Chemicals. [4-^{14}C]-Androst-4-ene-3,17-dione (androstenedione; spec. act. 59 mCi/mmol) was obtained from Amersham Australia (Sydney, N.S.W.).Testosterone, unlabelled androstenedione, 6β-hydroxy- and 16α-hydroxyandrostenedione, as well as all biochemicals, were purchased from Sigma Chemical Company (St Louis, MO). 7α-Hydroxyandrostenedione was obtained from Professor D. N. Kirk of the MRC Steroid Reference Collection, Queen Mary's College (London, U.K.). 16β-Hydroxyandrostenedione was prepared enzymatically by the action of 3β-hydroxysteroid dehydrogenase (Sigma Chemical Co.) on 3β,16β-dihydroxyandrost-5-ene-17-one (MRC Collection) as described by Talalay and Dobson [13]. Other

steroid standards were obtained from either Sigma, the MRC Collection, or Steraloids, Inc. (Wilton, NH).

7-Ethoxy- and 7-hydroxycoumarin were purchased from Aldrich Chemical Co. (Milwaukee, WI), and aminopyrine was obtained from Sigma. Aniline was obtained from Ajax Chemicals (Sydney, N.S.W., Australia) and was redistilled from zinc dust before use.

Clotrimazole and miconazole nitrate were purchased from Sigma. Ketoconazole was generously donated by Janssen Pharmaceutica (Australia) Pty. Ltd. (French's Forest, N.S.W.), econazole nitrate by Squibb—Australia (Noble Park, Victoria), and Dazmegrel and Dazoxiben by Pfizer Central Research (Sandwich, Kent, U.K.). The structures of these drugs are shown in Fig. 1.

Solvents and miscellaneous chemicals were from Ajax Chemicals, and were at least analytical reagent grade.

Animals. Male Wistar rats (250–300 g) were obtained from the animal house of the Institute of Clinical Pathology and Medical Research at Westmead Hospital. Animals were held under conditions of constant temperature (22°), humidity, and lighting (12 hr dark–light cycle). Hepatic microsomal fractions were prepared as described elsewhere [14].

Optical difference spectroscopy. Cytochrome P-450 was measured according to the method of Omura and Sato [15], using an Aminco-Chance DW-2a spectrophotometer and employing an extinction coefficient of 91 $mM^{-1}\,cm^{-1}$ for the ferrous P-450/carbonyl spectral complex.

Difference spectra were measured at 37° in an Aminco-Chance DW-2a spectrophotometer using 1-cm cuvettes containing 1-ml aliquots of microsomal suspensions (0.75 mg microsomal protein/ml) in potassium phosphate buffer (0.1 M, pH 7.4). Test compounds were added to the sample cuvette in microlitre quantities in dimethylformamide and the difference spectra were recorded between 380 and 500 nm; an equal volume of solvent was added to the reference cuvette. Spectral dissociation constants (K_s) and maximal spectral changes (ΔA_{max}) were determined by established procedures [16].

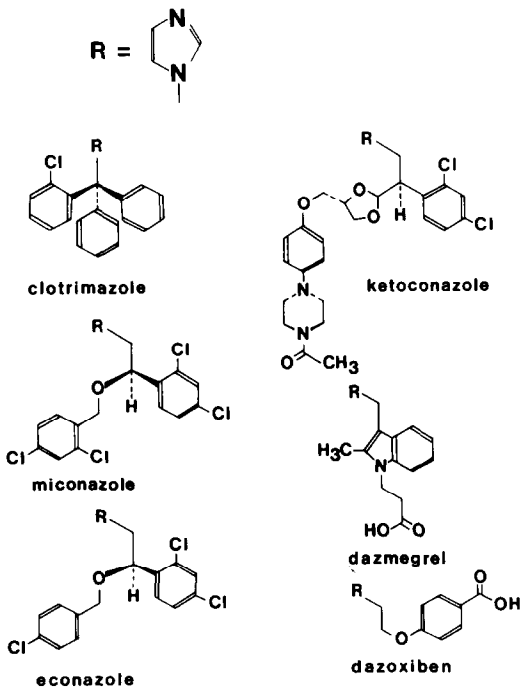

Fig. 1. Structures of antithrombitic and antimycotic imidazoles.

Androstenedione hydroxylase activity. Microsomal androstenedione hydroxylase activity was assayed as described previously [17]. Each incubation (4.0 ml final volume) contained 0.75 mg microsomal protein/ml, an NADPH-generating system (4 mM glucose 6-phosphate, 1 mM NADP, and 1 unit glucose 6-phosphate dehydrogenase) and androstenedione (final concentration 0.175 mM). Reactions were initiated by the addition of NADP and were terminated after 10 min by the addition of 5.5% zinc sulphate. The chloroform extract of the resultant supernatant was applied to TLC plates (silica gel 60, F_{254} type, 20 × 20 cm × 0.25 mm thickness, and activated 15 min at 100° before use; E. Merck, Darmstadt, F.R.G.). Plates were developed twice in the solvent system ($CHCl_3$: ethyl acetate 1:2, v/v) as described by Waxman *et al.* [2]. Zones corresponding to hydroxylated androstenedione standards were visualised under UV light and scraped into vials for scintillation spectrometry (Aquasol scintillant, New England Nuclear, Boston, MA).

Inhibitors were introduced into reaction incubations in 50 μl litres of dimethylformamide (final solvent concentration 1.25%); solvent alone was added to control incubations. This concentration of solvent produced 24–30% inhibition of the steroid hydroxylase pathways. Plots of log inhibitor concentration vs percent of control activity were constructed from mean percent inhibition data at 3 different inhibitor concentrations. Each point was the mean of 2–4 individual estimates from separate incubations. The mean standard error of hydroxyandrostenedione metabolite formation was 5.1%.

Other mixed-function oxidase activities. Aminopyrine *N*-demethylase activity was measured as described previously [18], 7-ethoxycoumarin *O*-deethylase activity by the spectrofluorometric procedure of Prough *et al.* [19], and aniline 4-hydroxylase activity by the method of Murray and Ryan [20]. Inhibition of each of these activities by the six imidazole drugs was assessed at least in duplicate at three different test concentrations.

Protein was determined according to Lowry *et al.* [21] using bovine serum albumin as standard.

RESULTS

Effects of antithrombitic imidazoles on microsomal steroid hydroxylase activities

Addition of the antithrombitic imidazoles dazoxiben and dazmegrel to microsomal incubations *in vitro* altered the relative activities of the four androstenedione hydroxylase pathways in a concentration-related fashion (Fig. 2). Dazmegrel (1 μM) elicited moderate inhibition of androstenedione 6β- and 16β-hydroxylation; the 16β-hydroxylase pathway was

also decreased to about 85% of control activity in the presence of 100 μM dazmegrel. Very slight inhibition of androstenedione 7α-hydroxylation was observed but the 16α-hydroxylase was either uninhibited or enhanced by dazmegrel. The pronounced enhancement of the 6β- and 16β-hydroxylase activities that was produced by 10 μM dazmegrel gave rise to the parabolic dose–response curve shown in Fig. 2.

Dazoxiben affected the four pathways of androstenedione hydroxylation in a similar manner to dazmegrel. Thus, over the concentration range 1–100 μM, dazoxiben produced enhancement of androstenedione 6β-, 16α- and 16β-hydroxylation and weak inhibition of 7α-hydroxylation when tested at concentrations of 10 and 100 μM (Fig. 2).

Effects of antimycotic imidazoles on microsomal steroid hydroxylase activities

In contrast to the situation observed with the antithrombitic agents, the four antimycotic imidazoles—clotrimazole, econazole, miconazole and ketoconazole—were potent inhibitors of the various pathways of androstenedione hydroxylation (Fig. 3). Clotrimazole was probably the most effective inhibitor of all pathways and the enzyme that appeared to be especially susceptible to inhibition was the 6β-hydroxylase (75% inhibition at 1 μM clotrimazole).

Econazole was probably the most effective steroid hydroxylase inhibitor after clotrimazole, followed by miconazole and with ketoconazole the least potent compound. In fact, ketoconazole was essentially non-inhibitory against all hydroxylase pathways when tested at a concentration of 1 μM. The imidazole concentration that permitted the best resolution of the various effects of the inhibitors was 10 μM. Thus, 10 μM concentrations of the four antimycotics elicited 30–90% inhibition of the steroid 6β- and 16β-hydroxylase pathways whereas 25–45% inhibition of the 7α-hydroxylase was noted. The response of the 16α-hydroxylase was dependent upon the inhibitor. Accordingly, clotrimazole (10 μM) inhibited this activity by 90%, econazole elicited 60% inhibition, miconazole 30% inhibition and ketoconazole did not inhibit the activity at all (Fig. 3).

Effects of antithrombitic and antimycotic imidazoles on microsomal xenobiotic metabolising enzymes

Three xenobiotic metabolising enzymes (aminopyrine *N*-demethylase, 7-ethoxycoumarin-*O*-deethylase and aniline 4-hydroxylase) were studied in rat hepatic microsomes in relation to their susceptibility to inhibition by each of the imidazole drugs (Table 1). The data that were obtained were quite similar to those for the inhibition of microsomal steroid hydroxylation. Thus, the four antimycotic imidazoles were markedly more potent inhibitors of xenobiotic oxidation than the two antithrombitic agents. Indeed, of the latter two drugs, only dazmegrel at a concentration of 100 μM effected notable inhibition (to 46–68% of control) of the test activities.

Three of the four antimycotic agents (econazole, miconazole and clotrimazole) were especially potent inhibitors of 7-ethoxycoumarin *O*-deethylase activity. Aniline 4-hydroxylase activity appeared to be least susceptible to inhibition by these agents. The remaining antimycotic drug, ketoconazole, was slightly less potent than the other three imidazoles

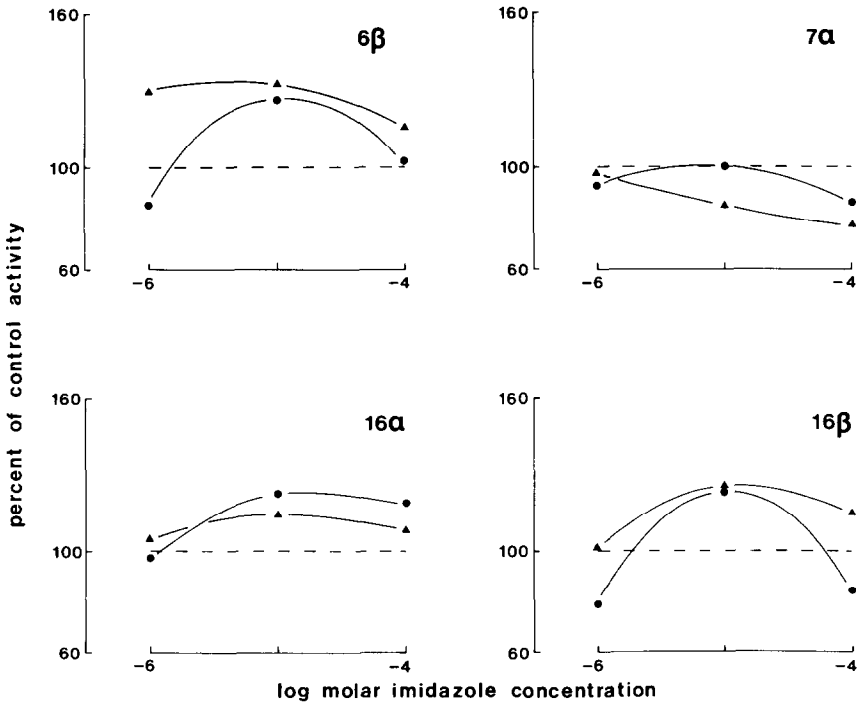

Fig. 2. Effect of antithrombitic imidazoles on rat hepatic microsomal androstenedione 6β-, 7α-, 16α- and 16β-hydroxylase activities *in vitro*. Key: (▲) dazoxiben and (●) dazmegrel.

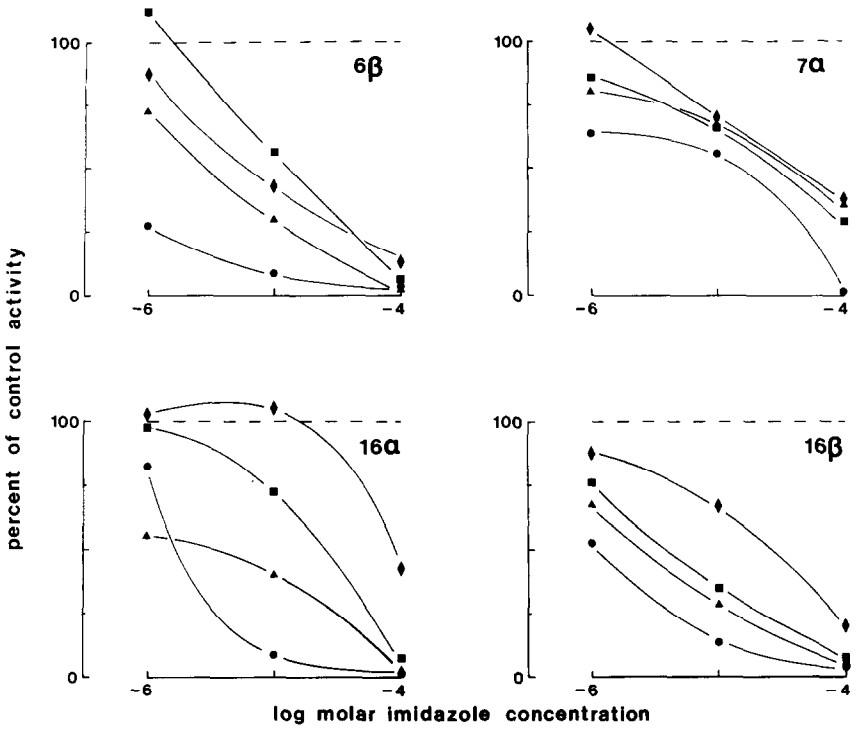

Fig. 3. Effect of antimycotic imidazoles on rat hepatic microsomal androstenedione 6β-, 7α-, 16α- and 16β-hydroxylase activities *in vitro*. Key: (◆) ketoconazole, (■) miconazole, (▲) econazole and (●) clotrimazole.

Table 1. Comparative potencies of antithrombitic and antimycotic imidazoles as inhibitors of three mixed-function oxidase activities in untreated rat hepatic microsomes

Imidazole	Concentration (μM)	Mixed-function oxidase activity† (% of control activity) ECOD	APDM	APH
Econazole	1	18	73	98
	10	12	46	78
	100	—*	28	58
Miconazole	1	31	83	103
	10	20	58	88
	100	—*	33	67
Clotrimazole	1	10	86	100
	10	8	35	85
	100	—*	28	66
Ketoconazole	1	35	97	99
	10	22	67	84
	100	9	35	75
Dazoxiben	1	59	111	101
	10	97	93	98
	100	86	87	94
Dazmegrel	1	95	105	97
	10	92	87	92
	100	46	53	68

* Activity too low to measure accurately.

† Control activities: ECOD (7-ethoxycoumarin *O*-deethylase): 240 pmole umbelliferone/min/mg; APDM (aminopyrine *N*-demethylase): 1.3 nmole formaldehyde/min/mg; APH (aniline 4-hydroxylase): 2.2 nmole 4-aminophenol/min/mg.

Table 2. Spectral binding characteristics of imidazole drugs in hepatic microsomes from untreated rats

Imidazole	K_s	ΔA_{max}	$\Delta A_{max}/K_s \times 10^{-3}$
Clotrimazole	0.26	4.55×10^{-2}	175
Econazole	0.54	5.19×10^{-2}	96
Miconazole	0.55	4.26×10^{-2}	78
Ketoconazole	0.73	4.02×10^{-2}	55
Dazmegrel	6.5	4.20×10^{-2}	6.6
Dazoxiben	21.0	2.08×10^{-2}	1.0

Data are means of two to four individual estimates; mean variation of 7.3%. Units are; K_s, μM; ΔA_{max}, absorbance units/nmole cytochrome P-450; $\Delta A_{max}/K_s$, absorbance units/nmole cytochrome P-450/M.

against each of the enzymes studied. Nevertheless, ketoconazole was a considerably more potent inhibitor of xenobiotic oxidation than either of the antithrombitic imidazoles.

Spectral binding interactions of imidazole drugs in rat hepatic microsomes

Binding studies were undertaken in order to assess the capacity of the six imidazoles to interact with oxidised microsomal P-450 (Table 2). All compounds elicited type II optical difference spectra with ferric P-450 and, in the case of the antimycotic imidazoles, the interaction was of very high affinity (K_s values in the range 0.26–0.73 μM). The extent of the binding interaction with ferric P-450 was also high for these compounds (ΔA_{max} values in the range $4.02–5.19 \times 10^{-2}$ absorbance units/nmole P-450). Dazmegrel and dazoxiben generated type II spectra with K_s values that were greater than those produced by the antimycotic drugs (but still of relatively high affinity compared with members of the benzimidazole series of inhibitors [16, 29]) and dazoxiben elicited a maximal absorbance change that was only about one-half that of the other compounds. The $\Delta A_{max}/K_s$ parameter, which is a measure of the efficiency of the binding reaction, was much larger for the four antimycotic imidazoles than for the antithrombitic agents.

DISCUSSION

The results of the present study show clearly that not all imidazole drugs are potent inhibitors of hepatic microsomal mixed-function oxidases. The antimycotic agents were quite potent inhibitors of steroid hydroxylase activities from untreated rat liver microsomes. This finding is in agreement with previous studies that assessed the capacity of similar compounds as inhibitors of steroid hydroxylases from phenobarbital-induced rat liver [22], vitamin D_3 hydroxylases from chick kidney [23] and placental aromatase activity [24]. However, it is clear from the present study that the antithrombitic drugs dazoxiben and dazmegrel are ineffective as inhibitors of the microsomal P-450-mediated steroid hydroxylases. In view of the recently established P-450 haemoprotein nature of the platelet thromboxane synthetase [25–27] it is apparent that very different factors influence the extent of inhibition of the hepatic and platelet P-450s. The antithrombitic and antimycotic imidazoles in the present study all possess *N*-arylalkyl substituents (Fig. 1). This structural feature is commonly associated with potent inhibition of mixed-function oxidase activity [6, 7] yet, clearly, the antithrombitic agents are extremely weak inhibitors of steroid hydroxylation. An important additional consideration is that the two antithrombitic imidazoles possess carboxylic acid functional groups within the *N*-substituent. The pK_a of the carboxylate function is about 4.5 so that less than one percent of these antithrombitic imidazoles would be present in the unionised form at physiological pH. P-450 has a high degree of hydrophobic character and ionised substrates and inhibitors would not be expected to interact significantly with the cytochrome. Despite this factor it appears that the antithrombitic imidazoles are at least able to interact with the haem iron of ferric P-450. As shown in Table 2, type II optical difference spectra of relatively high affinity were observed after the addition of dazmegrel and dazoxiben to oxidised microsomal fractions. One interpretation is that an imidazole ring nitrogen atom may interact with the P-450 haem iron even though the *N*-substituent has insufficient hydrophobic character to interact effectively with apoprotein binding sites that have been proposed to exist adjacent to the haem moiety [6, 28, 29].

Despite its non-potency as an inhibitor of the microsomal steroid hydroxylases, dazmegrel is an effective inhibitor of human platelet thromboxane synthetase [9, 30]. Thus, this compound clearly possesses the structural characteristics necessary for inhibitory binding to thromboxane synthetase in the presence of substrate (e.g. arachidonic acid). It is possible therefore that ionised groups may be coordinated within the active site of P-450-thromboxane synthetase. Alternately, the active site of platelet P-450 may contain a binding region of relatively high hydrophilicity. The hepatic P-450s have a quite low substrate specificity and hydrophobic character is a major determinant of substrate [28] and inhibitor [6, 29] interactions. It is possible that subtle differences exist between these P-450s and that responsible for platelet thromboxane synthetase activity. Imidazoles that are potent inhibitors of the hepatic microsomal P-450s are usually also effective inhibitors of the platelet P-450 system [12]. However, the reverse is not necessarily true, as indicated by the present data that demonstrate the lack of inhibition of microsomal steroid hydroxylases by thromboxane synthetase inhibitors.

Whereas individual microsomal xenobiotic oxidation pathways are often catalysed by more than a single P-450, the same is not true of steroid hydroxylation. The formation of each hydroxylated metabolite of androstenedione in untreated rat liver microsomes has been attributed to the activity of a single P-450. Thus, steroid 6β-, 7α-, 16α- and 16β-hydroxylations are catalysed respectively by P-450s PCN-E, UT-F, UT-A and PB-B [3] (according to the nomenclature of Guengerich). Steroid hydroxylase activities were therefore used in the present study to provide information regarding the relative susceptibility of these P-450s to inhibition by the imidazole drugs of interest. The phenobarbitone-inducible P-450s PB-B and PCN-E (the 16β- and 6β-

hydroxylases, respectively) appeared relatively more susceptible to inhibition by the antimycotic imidazoles. P-450 UT-F-mediated 7α-hydroxylation was relatively resistant to inhibition by these agents, whereas the response of P-450 UT-A-mediated androstenedione 16α-hydroxylation was dependent upon the specific inhibitor.

Steroid probes remain useful in the characterisation of P-450 isozymes that are subject to preferential inhibition by imidazoles and other agents. Although most of the antimycotic imidazoles are uniformly potent inhibitors of steroid hydroxylase pathways, and therefore multiple P-450s, other inhibitors could possess greater selectivity. Identification of those P-450s that are especially sensitive to inhibition by various therapeutic agents will ultimately provide valuable information concerning the drug metabolism pathways that are most susceptible to modulation during multiple drug therapy. Furthermore, the mechanisms of drug interactions may eventually be rationalised by this approach.

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