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A COMPARATIVE STUDY OF 1-SUBSTITUTED IMIDAZOLE AND 1,2,4-TRIAZOLE ANTIFUNGAL COMPOUNDS AS INHIBITORS OF TESTOSTERONE HYDROXYLATIONS CATALYSED BY MOUSE HEPATIC MICROSOMAL CYTOCHROMES P-450

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Abstract—Three imidazole antifungal agents, ketoconazole, miconazole and tioconazole, and a group of structurally related 1-substituted imidazole and 1,2,4-triazole compounds were evaluated as inhibitors of the oxidative metabolism of testosterone catalysed by mouse hepatic microsomal cytochromes P-450. Spectroscopic studies showed that both imidazoles and triazoles interacted with ferric cytochrome P-450 in hepatic microsomes to produce type II difference spectra which could be distinguished by their different absorbance maxima; 429–430 nm and 425–426 nm respectively. Compound 4, which possesses both types of functional group, produced a spectrum which resembled that of imidazole compounds, indicating that the imidazole moiety had a higher affinity than the triazole for the haem of cytochromes P-450 present in microsomes. The test compounds differentially inhibited regio- and stereo-specific testosterone metabolism and the pattern of inhibition varied with the 1-substituent on the azole ring. Ketoconazole was a potent inhibitor of testosterone 6β-hydroxylation (IC_{50} 0.08 μM) but was considerably less active against other hydroxylations and 17β-oxidation to androstenedione (IC_{50} range 13 to >100 μM). In contrast, tioconazole (IC_{50} range 0.18 to 3.3 μM) and miconazole (IC_{50} range 0.15 to 10 μM) were relatively non-selective. Compounds 1 and 2, which differed from each other only in the type of azole ring, were most active against 16β-hydroxylation. The triazole analogue (compound 2) was a significantly more potent inhibitor of 16β-hydroxylation than the imidazole (compound 1), equipotent against androstenedione formation and less active against the other hydroxylations. Two relatively polar bis-azole analogues (compounds 3 and 4) were most active against androstenedione formation; however, in general they were less inhibitory than the lipophilic azoles. We conclude that azole antifungal agents of differing structure show different patterns of selective interaction with cytochromes P-450, a phenomenon primarily dependent on the 1-substituent on the azole ring, but also modulated to a lesser extent by the type of azole ring (imidazole or triazole).

The group of azole compounds, comprising 1-substituted imidazole and 1-subsituted 1,2,4-triazole derivatives, are recent introductions into antifungal therapy [1]. Their mode of action involves inhibition of the cytochrome P-450 dependent 14α-demethylase activity required in the conversion of lanosterol to ergosterol [2, 3], an essential steroid component of fungal cell membranes. A number of clinically used imidazole antifungal drugs including ketoconazole,‡ miconazole and clotrimazole, have been reported to inhibit the mammalian cytochrome P-450 activities involved in xenobiotic metabolism [4, 5] and also steroidogenesis in adrenal, gonadal and placental tissues [6–9]. However, azole antifungal compounds are not equipotent inhibitors of all cytochrome P-450 linked activities; ketoconazole differentially inhibits the cytochrome P-450 mediated steroid hydroxylations in rat hepatic microsomes [10] and we have recently shown that compound 1 (Fig. 1) exhibits selectivity in the inhibition of ethoxyresorufin O-deethylases in mouse hepatic microsomes [11]. Furthermore, 1-phenylimidazole has been reported to be a potent inhibitor of aromatic hydrocarbon hydroxylation (AHH) in microsomes from phenobarbitone-induced rats but has little activity against AHH in 3-methylcholanthrene-induced animals [12, 13].

The objective of this investigation was to evaluate a series of 1-substituted azole compounds (Fig. 1) as inhibitors of cytochromes P-450 catalysing the

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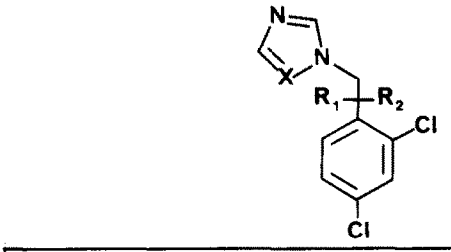
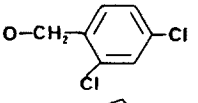
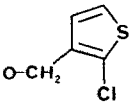
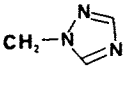
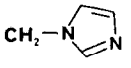
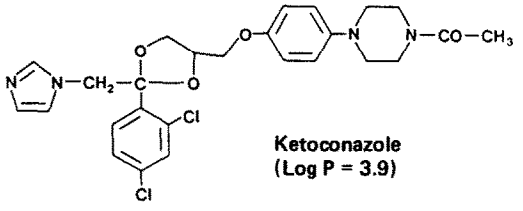
‡ Abbreviations used: testosterone, 17β-hydroxyandrost-4-ene-3-one; androstenedione, androst-4-ene-3,17-dione; OHT, hydroxytestosterone; TMS, trimethylsilyl; IC_{50}, concentration of test compound producing 50% inhibition; triazole, 1,2,4-triazole; log *P*, log of octanol/water partition coefficient; hepes, N-2-hydroxyethylpiperazine-N′-ethanesulphonic acid; TEMED, *NN′N*-tetramethyl-1,2-diaminoethane; ketoconazole, (±)-cis-1-acetyl-4-[4-[1[2-(2,4-dichlorophenyl)-2-(1H-imidazole-1-yl-methyl)-1,3-dioxalan-4-yl]methoxy]-phenyl]piperazine; miconazole, (±)-1-[2-(2,4-dichlorophenyl)-2-[(2,4-dichlorophenyl)methoxy]ethyl]-1H-imidazole; tioconazole, (±)-1-[2-(2,4-dichlorophenyl)-2-[(2-chlorothien-3-yl)methoxy]ethyl-1H-imidazole; compound 1, (±)2-(2,4-dichlorophenyl)-1-(1H-imidazol-1-yl)octan-2-ol; compound 2, (±)2-(2,4-dichlorophenyl)-1-(1H-1,2,4-triazol-1-yl)octan-2-ol; compound 3, 2-(2,4-dichlorophenyl)-1,3-bis(1H-1,2,4-triazol-1-yl)propan-2-ol; compound 4, (±) 2-(2, 4-dichlorophenyl)-1-(1H-imidazole-1-yl)-3-(1H-1,2,4-triazol-1-yl)propan-2-ol.



Compound	X	R_1	R_2	Log P
Miconazole	C		H	5.7
Tioconazole	C		H	4.7
1	C	$(CH_2)_5CH_3$	OH	5.2
2	N	$(CH_2)_5CH_3$	OH	4.6
3	N		OH	1.1
4	N		OH	1.6



Ketoconazole
(Log P = 3.9)

Fig. 1. Structures and log P values for the azole compounds used in this study. Log P values were determined by HPLC as described in Materials and Methods.

oxidation of testosterone in mouse hepatic microsomes, with a view to obtaining information on structure activity relationships. Testosterone was selected as the probe substrate because many cytochromes P-450 hydroxylate it at regio- and stereo-specific sites [14, 15]. Consequently, by determining the rates of production of individual testosterone metabolites it was possible to rapidly "fingerprint" the effect of the inhibitors on a range of isoenzymes.

MATERIALS AND METHODS

Ketoconazole and miconazole were supplied by Janssen Pharmaceutica N.V. (Beerse, Belgium). Tioconazole and compounds 1 to 4 (Fig. 1) were supplied by Pfizer Central Research (Sandwich, Kent, U.K.), and correspond to UK-20349, UK-39671, UK-46245, UK-47265 and UK-39896 respectively. All azoles except compound 3 were racemic mixtures of two enantiomers. [4-^{14}C]-testosterone (58 mCi/mmol) was obtained from Amersham International plc (Amersham, U.K.). 2α-, 2β-, 6α-, 6β-, 7α-, 15α-, and 16α-hydroxytestosterones were supplied by the MRC Steroid Reference Collection (London, U.K.). 16β-Hydroxytestosterone was obtained from Steraloids Inc. (P.O. Box 310, Wilton, NH 03086, U.S.A.). Silica gel TLC plates (13181) were purchased from Eastman Kodak (Liverpool, U.K.). Bond-Elut C-18 cartridges were obtained from Analytichem Ltd. (Luton, U.K.). Sil-A silylating reagent was purchased from Sigma (Poole, U.K.).

All other reagents were of the highest grade available and were purchased from commercial supply houses.

Preparation of microsomes. Female Swiss Ham CD1 mice (body weight 20–25 g; Charles River, Manston, U.K.) were killed by cervical dislocation and the livers transferred to ice cold saline (0.9% w/v). Microsomes were prepared from livers as described by Nakamura and Ueda [16], and stored at −80° as a suspension (approx. 10 mg protein/ml) in potassium phosphate buffer (0.1 M, pH 7.4) containing 20% (v/v) glycerol. The storage period

did not exceed 4 months, during which the cytochrome P-450 concentration was stable.

The concentration of protein in microsomes was determined by the method of Lowry *et al.* [17] using bovine serum albumin as a standard, and cytochrome P-450 concentration was determined according to Omura and Sato [18]. NADPH cytochrome c (P-450) reductase activity was measured by the method of Strobel and Dignam [19] using a microsomal protein concentration of 0.01 mg/ml.

Spectrophotometric binding studies. Microsomal suspensions were diluted with potassium phosphate buffer (0.1 M; pH 7.4) to give a final cytochrome P-450 concentration of 1 μM. Samples (5 ml) were divided equally between two quartz cuvettes and a base line recorded from 350–500 nm using a Kontron Uvikon 810 spectrophotometer. Difference spectra were recorded after the addition of test compound dissolved in methanol to the sample cuvette and an equal volume of solvent to the reference. The final concentration of methanol did not exceed 1% (v/v).

Testosterone hydroxylase assay. Studies were performed using a single batch of microsomes (prepared from the pooled livers of 20 female mice) which contained 1.0 nmol cytochrome P-450/mg protein. The testosterone hydroxylase activity in stored microsomes was stable for at least 4 months.

The reaction mixture (final volume 1 ml) contained mouse hepatic microsomes (0.5 μM cytochrome P-450), hepes-Na buffer (50 mm; pH 7.4), magnesium chloride (5 mM), manganese chloride (5 μM), isocitrate (5 mM), isocitrate dehydrogenase (0.5 units/ml), [4-^{14}C]-testosterone (100 μM; 0.09 μCi/ml) and test compound or vehicle (10 μl of methanol). After incubation at 37° for 1 min the reaction was initiated by addition of NADPH to give a final concentration of 0.5 mM. After a further 10 min incubation the reaction was stopped by addition of methanol (1 ml) followed by vigorous mixing. The reaction mixture was placed on ice for 5 min and the precipated protein then removed by centrifugation (2000 *g*; 10 min). The supernatant was diluted with an equal volume of water and testosterone metabolites and unmetabolised testosterone were extracted as described by Darby *et al.* [20] using a Bond–Elut C-18 cartridge. Radioactivity was recovered with an efficiency of 93 ± 4% (N = 12); the recoveries of major metabolites and testosterone were similar.

The extract was evaporated to dryness under a stream of nitrogen, redissolved in methanol (50 μl) and a sample containing about 10^5 dpm was applied in a 1.5 cm band onto silica gel TLC plates which had been activated at 100° for 30 min. Standards were applied and the plates developed three times in solvent system A (dichloromethane : acetone; 75 : 25, v/v). ^{14}C-Labelled metabolites were located by autoradiography and standards by fluorescence quenching. Individual bands were cut from the plate and radioactivity was quantified by liquid scintillation counting. Radioactivity was recovered with an efficiency of 92 ± 13% (N = 24).

For assessment of homogeneity, metabolites were eluted from TLC plates with dichloromethane : acetone (2 : 1, v/v), and the extract evaporated to dryness and redissolved in methanol (50 μl) prior to TLC analysis with three developments using solvent system B (dichloromethane : ethanol; 20 : 1, v/v) or solvent system C (cyclohexane : ethylacetate; 1 : 2, v/v).

Identification of testosterone metabolites. Testosterone metabolites were recovered from TLC plates and silylated essentially as described by Gustafsson [21] using Sigma Sil-A silylating reagent. The TMS derivatives were analysed by capillary GC mass spectrometry and metabolites identified on the basis of their retention times and mass ion profiles by reference to those of standards.

Determination of IC_{50} *values.* IC_{50}s were calculated from sigmoid curves fitted to inhibitor-response data from two to three experiments using the constrained curve fitting procedure of De Lean *et al.* [22]. The values of maximum (control) and minimum (fully inhibited) activities were set at 100% and 0% respectively unless these constraints were inconsistent with the data. The test azoles did not stimulate any testosterone oxidation, however, approximately 18% of 17β-oxidase activity was not inhibitable (see Results) and therefore the minimum was set at 18% for this reaction. The SEM of IC_{50}s represents the predicted limits of inter-experimental variation calculated from the error in curve fit. Statistical significance of differences between IC_{50} values was determined using the *F*-test procedure described by De Lean *et al.* [22].

Determination of log P *values.* Log *P* values for azoles were determined using HPLC in a modification of the procedure described by Terada [23]. A Hichrom S5 ODS2 reverse phase column (Hichrom, Reading, Berks., U.K.) was used with a mobile phase (flow rate 1 ml/min) consisting of methanol : TEMED (0.1 M; adjusted to pH 7.4 with phosphoric acid) in proportions varying from 90 : 10 to 40 : 60. Retention times of test azoles were determined at four concentrations of methanol and capacity factors (k') and derived capacity factors in the absence of methanol (k_0) were calculated as described by Terada [23]. Using azole standards of known log *P*, Eqn (1) was derived which allowed the log *P* of test compounds to be calculated from the log k_0:

$$\log P = (1.11 \log k_0) - 1.44. \quad (1)$$

RESULTS

Spectroscopic studies

The structures of the test azoles and their log *P* values are shown in Fig. 1. Each azole produced a type II binding difference spectrum with hepatic microsomes; however, differences in the characteristics of the spectra were evident. Compounds with a single imidazole gave spectra with a maximum absorbance at 429–431 nm, an isobestic point at 418–420 nm and a broad minimum from 390–408 nm (Fig. 2; compound 1). Spectra for compounds 2 (Fig. 2) and 3 (data not shown), which contain only triazole moieties, showed maxima and isobestic points at 426–427 nm and 417–418 nm respectively, with minima at 390–408 nm. The spectrum produced by compound 4, which contained both types of azole group, resembled those of the imidazoles in having a maximum at 429–430 nm, and an isobestic point at

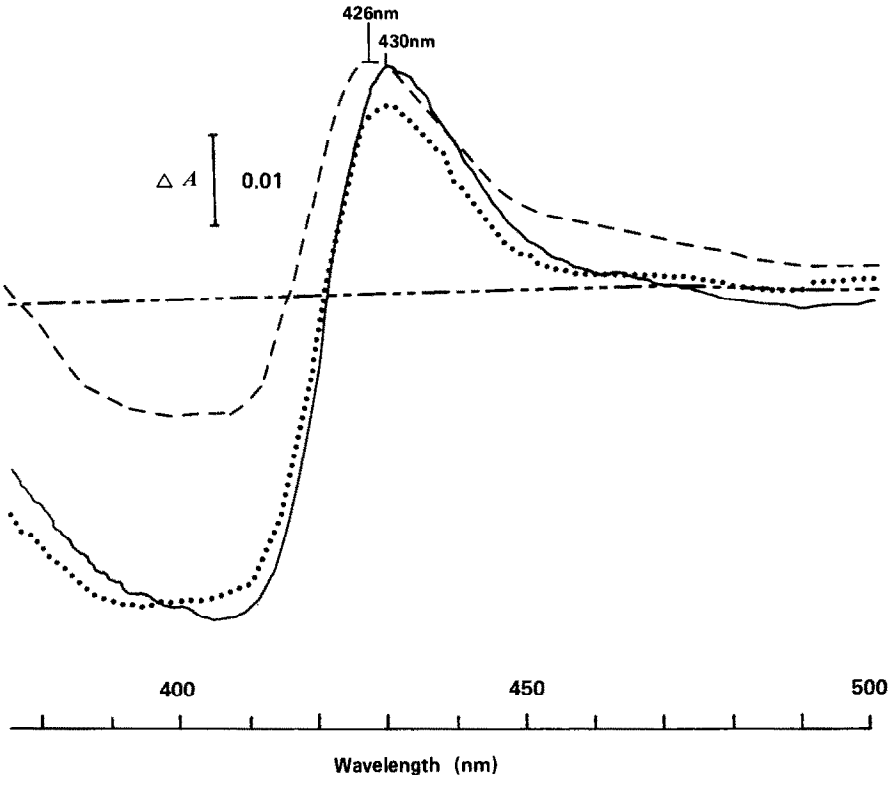


Fig. 2. Difference spectra produced by test azoles with mouse hepatic microsomes. Microsomes were suspended in potassium phosphate buffer (0.1 M; pH 7.4) at a cytochrome P-450 concentration of 1 μM and the azole compounds were added to give 10 μM in the sample cuvette. Key: (——) compound 1; (– – –) compound 2; (· · ·) compound 4.

418–419 nm (Fig. 2). The high affinity of the test compounds for cytochromes P-450, in some cases resulting in stoichiometric binding [Ballard, unpublished observations], precluded an accurate assessment of the free concentration of ligand and consequently apparent dissociation constants could not be determined.

Separation and identification of testosterone metabolites

Figure 3 shows an autoradiogram of TLC separation of the metabolites resulting from incubation of mouse hepatic microsomes with ^{14}C-testosterone. When NADPH was omitted no metabolites were formed (Fig. 3, lane a) the small amounts of androstenedione and a contaminant co-chromatographing with Band 4 were also present at similar levels in the radiolabelled substrate. Rates of androstenedione formation were corrected for the presence of the contaminant. In the presence of NADPH, 10 discrete metabolite bands were resolved (Fig. 3; lane b). The five major hydroxytestosterone metabolites and androstenedione which formed the focus of inhibition studies were at least 80% pure (Table 1). None of the test azoles inhibited androstenedione formation completely. The residual activity (18%) was probably due to non-cytochrome P-450 17-oxidoreductase activity which has been reported to be insensitive to a number of antifungal imidazoles [10]. Of the remaining metabolites, bands 1 and 2 were not homogeneous and bands 3 and 4 did not absorb UV light and therefore were probably reduced metabolites produced by non-cytochrome P-450 enzymes.

The major testosterone metabolites were, in descending order of abundance, 6β-OHT, 7α-OHT, 16α-OHT, 15α-OHT, androstenedione, and 16β-OHT (Table 1). Other hydroxytestosterones, identified in bands 1 and 2, were 2β-OHT, 15β-OHT and 6α-OHT. These metabolites have all previously been reported as products of female mouse (C57B1/6J) hepatic microsomes [24] and 6β-OHT has consistently been found to be the major product [25, 26]. Formation of all hydroxytestosterones was linear with time for at least 10 min, and with cytochrome P-450 concentration over the range 0.25–1 μM. The rate of formation of androstenedione marginally decreased after 5 min, probably due to its further oxidation by cytochromes P-450 [27].

Inhibition studies

Each test azole inhibited hydroxylation of testosterone and androstenedione formation. Sigmoid dose–response curves described the experimental data satisfactorily, with at least 89% of the data variance being explained by the fit. Typical curves are shown in Fig. 4 and IC_{50} values for each compound are given in Table 2. The azoles were all

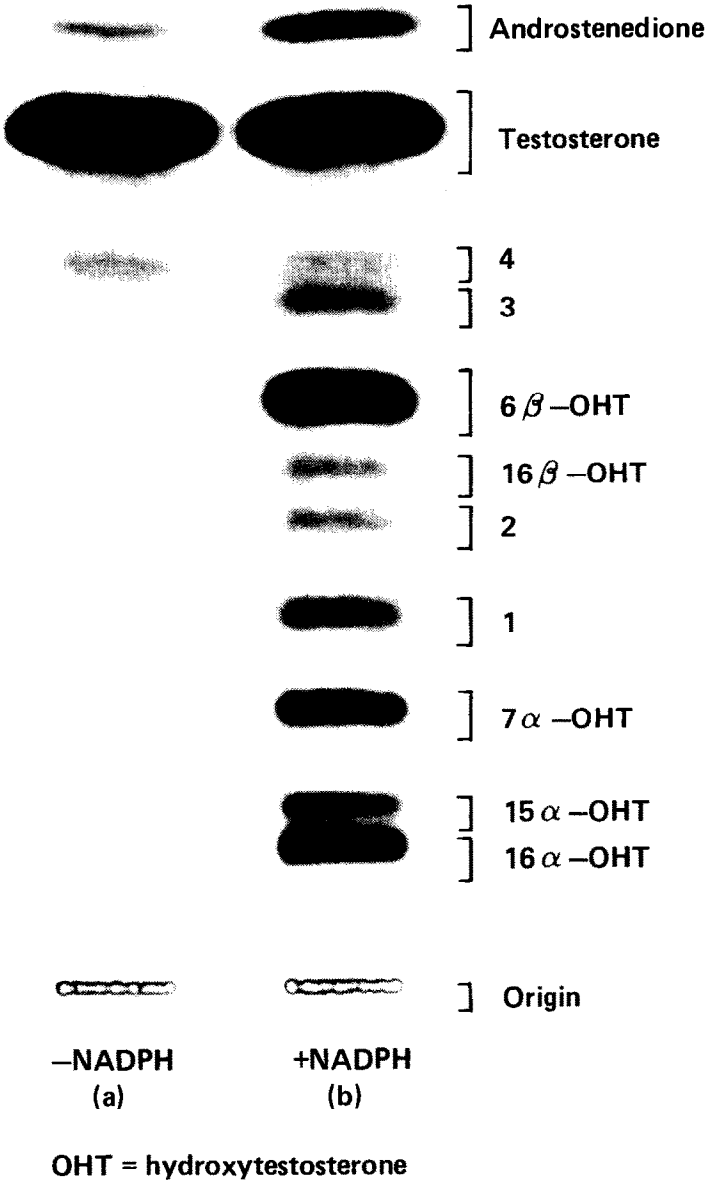


Fig. 3. Autoradiogram of a TLC separation of testosterone metabolites produced by mouse hepatic microsomes. [4-^{14}C]-Testosterone (100 μM) was incubated with microsomes (0.5 μM cytochrome P-450) in the presence or absence of NADPH for 10 min at 37°. Metabolites were extracted and separated by TLC using solvent system A (dichloromethane : acetone; 75:25 (v/v)) as described in Materials and Methods.

potent inhibitors of 6β-hydroxylation of testosterone and, with the exception of ketoconazole, androstenedione formation. Comparison of the IC_{50} values (Table 2) shows that the compounds differed markedly in their inhibition of other testosterone oxidations. Ketoconazole was a potent selective inhibitor of 6β-hydroxylation ($IC_{50} = 0.08\ \mu M$), while androstenedione and 15α-OHT formation were insensitive ($IC_{50} > 100\ \mu M$). Tioconazole was relatively non-selective and gave IC_{50}s in the range 0.18 μM to 3.3 μM for all reactions. Miconazole, which differs from tioconazole in the replacement of a 2-chlorothienyl group with a 2,4-dichlorophenyl group, produced similar IC_{50}s to tioconazole for each metabolite with the exception of 15α-OHT.

The effects of substituting a triazole for an imidazole group were examined by comparing compounds 1 and 2. The order of increasing sensitivity of testosterone oxidations to inhibition by these analogues was similar. However, the triazole (compound 2) was a significantly ($P < 0.01$) more potent inhibitor of 16β-hydroxylation, while the imidazole (compound 1) was more potent against the other hydroxylations. Both analogues showed similar activity against androstenedione formation. Replacement of the hexyl group of compound 2 with

Table 1. Major metabolites of testosterone produced by mouse hepatic microsomes: purifities following resolution by TLC and rates of formation*

Metabolite	% Purity†	Rate of production‡ (nmol/min/nmol cytochrome P-450)
16α-OHT§	81	0.50 ± 0.07
15α-OHT	91	0.26 ± 0.03
7α-OHT	100	0.60 ± 0.06
16β-OHT	82	0.17 ± 0.03
6β-OHT	93	1.36 ± 0.14
Androstenedione	100	0.24 ± 0.03

* Metabolites, generated by incubation of [4-^{14}C]-testosterone with mouse hepatic microsomes, were separated by TLC using solvent system A, and quantified by liquid scintillation counting. Purities of metabolites were determined by further TLC analysis using solvent systems B and C, and their indentities by capillary GC mass spectrometry as described in Materials and Methods

† Lowest of two values obtained using TLC solvent systems B and C.

‡ Rates are means (±SD) of 26 to 30 determinations.

§ OHT: hydroxytestosterone.

a more polar methylene-triazole moiety (compound 3) resulted in a marked reduction in inhibitory potential and a change in the pattern of inhibition; thus androstenedione production was most sensitive to compound 3. Compound 4, an analogue of compound 3 in which one of the triazole groups is replaced by an imidazole, produced a qualitatively similar inhibition profile to compound 3 but was consistently a more potent inhibitor.

The possibility that inhibition of testosterone oxidations might involve inhibition of electron transport to cytochromes P-450 was tested by measuring NADPH cytochrome C (P-450) reductase activity. At a concentration of azole equivalent to the maxi-

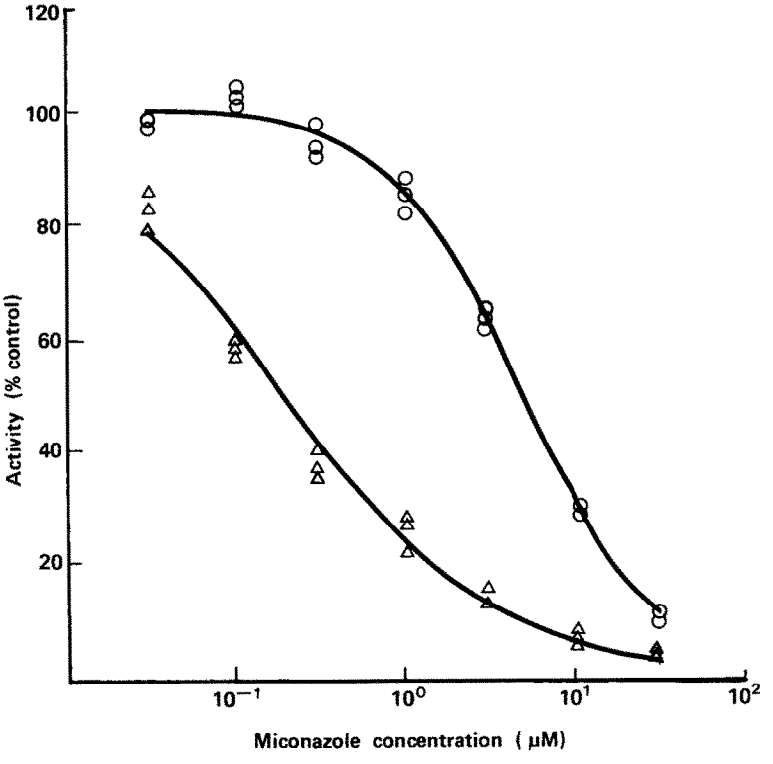


Fig. 4. Dose–response curves showing the inhibition of testosterone 6β- and 7α-hydroxylase activities by miconazole. Testosterone (100 μM) was incubated with mouse hepatic microsomes (0.5 μM cytochrome P-450) and a NADPH regenerating system in the presence of various concentrations of miconazole. The rates of formation of metabolites were determined as described in the text and sigmoid curves were fitted using the procedure of De Lean *et al.* [22]. Key: (△) testosterone 6β-hydroxylase; (○) testosterone 7α-hydroxylase.

Table 2. Summary of data for the inhibition by imidazole and triazole compounds of site-specific metabolism of testosterone catalysed by mouse hepatic microsomes*

Testosterone metabolite	IC_{50} (μM) ± SEM						
				Compound			
	Ketoconazole	Miconazole	Tioconazole	1	2	3	4
16α-OHT†	31 ± 5	4.0 ± 0.4	2.7 ± 0.3	3.3 ± 0.6	10 ± 2	280 ± 50	75 ± 19
15α-OHT	>100	10 ± 1	1.8 ± 0.2	15 ± 3	>30	320 ± 30	56 ± 9
7α-OHT	13 ± 1	5.1 ± 0.2	3.3 ± 0.3	15 ± 3	>30	340 ± 40	71 ± 11
16β-OHT	22 ± 3	0.8 ± 0.1	0.64 ± 0.06	0.16 ± 0.03	0.05 ± 0.02	67 ± 16	5 ± 1
6β-OHT	0.08 ± 0.01	0.20 ± 0.02	0.39 ± 0.03	0.21 ± 0.02	0.55 ± 0.08	9 ± 1	1.1 ± 0.2
Androstenedione	>100	0.15 ± 0.01	0.18 ± 0.02	0.20 ± 0.02	0.20 ± 0.03	1.4 ± 0.2	0.50 ± 0.09

* [4-^{14}C]-Testosterone (100 μM) was incubated with mouse hepatic microsomes (0.5 μM cytochrome P-450) for 10 min at 37° in the presence of the test compound at a range of concentrations. The rates of formation of testosterone metabolites were determined described in the text. IC_{50} values were calculated from sigmoidal dose–response curves fitted, by the procedure of DeLean *et al.* [22], to data from two or three experiments, each with at least seven concentrations of the azole.

† OHT: hydroxytestosterone.

mum used in IC_{50} determinations there was no significant inhibition of this enzyme (data not shown).

DISCUSSION

It is clear from studies in our laboratory [11, 28] and others [4–10, 13] that 1-substituted imidazole compounds, particularly the lipophilic azole antifungal agents, are potent inhibitors of cytochrome P-450 mediated reactions. However, few investigations have focused on the specificity of inhibition of individual forms of cytochrome P-450 by azoles with differing substitution pattern. The results of the studies reported here clearly demonstrate that, while the azole is the key factor in the inhibitory interaction with the haem of cytochromes P-450, the N-substituent is the major determinant of which isoenzyme will be most affected. It is now generally accepted that the interaction of nitrogen ligands with the haem of cytochromes P-450 is stabilised by binding of the non-ligating portion at a lipophilic site [29]. The present study indicates that for different isoenzymes this lipophilic site shows different binding characteristics and consequently lends support to the view that it is involved in substrate binding [30, 31].

Type II difference spectra, which are characteristic of the interaction of nitrogenous ligands with the haem of cytochromes P-450 [32], were obtained with all of the compounds tested. Spectral differences were observed between analogues in which the only difference was the replacement of an imidazole moiety with a triazole, but there were no marked alterations when the 1-substituent was changed. The haem ligating group therefore appears to dominate the characteristics of difference spectra. Since the spectrum produced by compound 4, containing both types of azole group, resembled those of the imidazoles it is probable that the imidazole group is a stronger ligand for the haem of isoenzymes constituting the majority of cytochrome P-450 in hepatic microsomes.

All of the compounds tested proved to be potent inhibitors of testosterone 6β-hydroxylation which in rat hepatic microsomes is catalysed by P-450 PB-2a and one or more closely related isoenzymes [33]; a cytochrome P-450 with similar properties has also been reported to be present in mouse hepatic microsomes [34]. This family of cytochromes P-450 therefore appears to show the least selectivity in terms of the azole inhibitor binding site. Major differences between compounds were, however, observed for the inhibition of other site-specific oxidations of testosterone. Tioconazole and miconazole, which are lipophilic imidazoles differing only in the replacement of a 2-chlorothienyl group with a 2,4-dichlorophenyl group were equipotent against the production of most metabolites and were the least selective compounds tested. However, since tioconazole was five-fold as potent as miconazole against 15α-hydroxylation, it is likely that the 2-chlorothienyl ring enhances interaction with the isoenzyme, P-450$_{15\alpha}$, which has been reported to be responsible for the majority of this activity in mouse hepatic microsomes [25].

At the other end of the selectivity spectrum, ketoconazole was a potent inhibitor of 6β-hydroxylation but a relatively poor inhibitor of the other oxidations. Sheets *et al.* [10], and Houston *et al.* [28] using hepatic microsomes from phenobarbitone-treated and untreated rats respectively, also found that ketoconazole was a more potent inhibitor of 6α-hydroxylation than of 16β- and 16α- hydroxylation. Consequently the selective interaction of ketoconazole with cytochromes P-450 catalysing 6β-hydroxylation is similar in mouse and rat.

The close structural series represented by compounds 1 to 4 serve to demonstrate that the nature of the heterocycle is relatively unimportant in determining the specificity of inhibition of different isoenzymes of cytochrome P-450, but may be a determinant of overall potency. Thus for pairs of compounds differing only in the nature of one azole group (compound 1 vs 2, and compound 3 vs 4) the ranking of sensitivity of testosterone oxidations to inhibition was similar. It is apparent, however, that generally the imidazole analogues are more potent inhibitors than the corresponding triazoles. This supports the conclusion from spectroscopic studies that the imidazole is, overall, a stronger ligand for the haem of cytochromes P-450 in hepatic microsomes. Nevertheless, 16β-hydroxylation was more sensitive to inhibition by the lipophilic triazole, compound 2, than by its imidazole analogue, compound 1, consequently the triazole may have higher affinity for some cytochrome P-450 isoenzymes.

The lipophilic azoles were generally more potent inhibitors of testosterone oxidations than the relatively polar bis-azoles, a result consistent with the report of Wilkinson *et al.* [35] that the inhibitory potency of alkylimidazoles against aldrin expoxidation was highly correlated with lipophilicity. However, it is likely that steric and structural features, rather than differences in lipophilic character, account for differences in the patterns of inhibition since tioconazole and compound 2, with similar log *P* values (Fig. 1), showed markedly different inhibition profiles. In addition the bis-azole, compound 3 ($\log P = 1.1$), was at least 70-fold as potent as ketoconazole ($\log P = 3.9$) against androstenedione formation. The low potency of ketoconazole against production of androstenedione was atypical for the group of compounds studied and, given its large size, steric factors may hinder its interaction with cytochrome(s) P-450 catalysing 17β-oxidation.

These studies show that the azole compounds exhibit a marked heterogeneity in their inhibition of cytochrome P-450 isoenzymes. As a consequence, azoles may prove useful tools in the further characterisation of cytochromes P-450. Indeed, with the present group of compounds it was found that none of the testosterone oxidations consistently correlated with any other in its sensitivity to inhibition and therefore supported the conclusion that each reaction was catalysed by different groups of cytochromes P-450 containing one or more isoenzymes. The selectivity of azoles could also benefit their development as novel therapeutic agents. Thus, for example, inhibitors of cytochromes P-450 involved in the steroidogenic pathways to testosterone and oestrogens are of interest as potential drugs for the treatment of prostatic and breast cancers respectively [36, 37], while some azole compounds have been shown to be effective cholesterol lowering agents and may have an application in the treatment of hypercholesterolaemia [38]. The important goal in the development of these agents will be to achieve selectivity of action against the specific forms of cytochrome P-450 catalysing the production of the target steroid(s).

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