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Some 1-[(Benzofuran-2-yl)methyl]imidazoles as Inhibitors of 17α-Hydroxylase:17, 20-lyase (P450 17) and Their Specificity Patterns

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Abstract

Four 1-[(benzofuran-2-yl)methyl]imidazoles (**1–4**) have been evaluated as in-vitro inhibitors of human testicular and bovine adrenal microsomal 17α-hydroxylase:17,20-lyase (P450 17) as potential anti-prostatic agents. Their specificity towards other steroidogenic and liver enzymes has been compared with that of ketoconazole.

All four compounds were inhibitors of the testicular enzyme (**2**, IC50 (concentration resulting in 50% inhibition) 0·185 μM; **4**, IC50 0·18 μM) but less potent than ketoconazole (IC50 0·03 μM). Towards bovine adrenal enzyme **2** and **4** were 35- and 31-fold more potent than ketoconazole (IC50 = 39·8 μM).

Compound **2** is a useful lead compound but although less potent than ketoconazole towards $P450_{SCC}$ and $P450_{11\beta}$, but not $P450_{C21}$, at the enhanced dose required for equivalent effects in-vivo on P450 17 it is likely that cortisol and aldosterone production will be affected to a greater extent than with ketoconazole.

In Western Europe prostate cancer is the most common malignant disease of the urinary tract and the second most frequent neoplasm in men (Denis 1991). It is the second most common disease causing death from cancer in men in the USA (Chiarodo 1991). Prostate cancer occurs largely among older men and is rare before the age of 50. It has a slow but progressive growth in most patients and it is estimated that 50% of patients who develop prostate cancer will die from the disease. Early prostatic cancer growth is androgen-dependent. In the prostatic epithelial cell (and peripheral tissue) testosterone is converted by the action of 5α-reductase to dihydrotestosterone the active intracellular androgen (Anderson & Liao 1968). Dihydrotestosterone is then believed to bind to a specific androgenic receptor protein which in this activated form binds to an acceptor site located on the DNA of nuclear chromatin and regulates transcription of specific target genes leading to a specific response of the cell. Therapies for prostatic cancer to remove androgen support are directed at: suppression of synthesis of testicular testosterone by orchidectomy (Denis 1991), use of LH–RH agonists (Crawford et al 1990), inhibition of 17α-hydroxylase:17, 20-lyase (P450 17) (Trachtenberg & Pont 1984) or 5α-reductase inhibition (Presti et al 1992); and blocking combination of the androgen with its receptors (anti-androgens) (Knuth et al 1984).

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In man testosterone is produced mainly in the testes by the action of P450 17 on pregnenolone, in the Δ^5-pathway, to give epi-androstenedione which is then isomerized and finally reduced by 17β-hydroxysteroid dehydrogenase. Although the adrenal gland secretes androstenedione and dehydroepiandrosterone these only account for approximately 5–10% of the total amount of circulating androgens, as a result of metabolic conversion to testosterone and dihydrotestosterone in the peripheral tissues or in the prostatic cell.

Ketoconazole (Figure 1), an imidazole derivative mainly used as an antimycotic agent, has been used in high dose for the treatment of prostatic cancer (Trachtenberg & Pont 1984; De Coster et al 1986). It reduces testosterone plasma levels by inhibiting testicular (and adrenal) P450 17 mainly in the 17α-hydroxylation step in man (Higashi et al 1987) but is an inhibitor of several other cytochrome P450 enzymes on the steroidogenic pathway (Sonino 1987). At the high doses used in therapy the principal side effect is gastric discomfort with nausea.

Its use is further limited by a thrice daily dosing schedule owing to rapid metabolism and liver damage in some patients (Lake-Bakaar et al 1987) leading to its withdrawal. These limitations have prompted the development of other agents with more acceptable profiles. Liarozole (Figure 1), an imidazole, also inhibits testicular but not adrenal enzyme (Vanden Bossche & Willemsens 1991) and similarly reduces testosterone levels in volunteers. Liarozole (and ketoconazole) inhibit the metabolism of retinoic acid, a cellular differentiating agent, to the inert 4-hydroxy derivative by specific cytochrome P450s leading to increased tissue and plasma endogenous retinoic acid levels (Vanden Bossche & Willemsens 1991) which might account for its anti-cancer action (De Coster et al 1996). Liarozole has been used clinically and results have been encouraging (Trachtenberg & Toledo 1991). 17-(3-Pyridyl)androsta-5,16-dien-3β-ol (CB7598; Figure 1) is a potent inhibitor ($K_i < 1$ nM) of human testicular enzyme (Potter et al 1995) and reduces circulating testosterone in the mouse and rat (as the acetate); it is now in early clinical trials (Barrie et al 1995). Other inhibitors include bifonazole, clotrimazole, miconazole, econazole (Ayub & Levell 1989), pyridylacetic acid derivatives (McCague et al 1990), bifluranol and its analogues (Barrie et al 1989), pyridyl-substituted tetralone derivatives (Wächter et al 1996) and 4-pregnene-3-one-20β-carboxaldehyde and its oxime (Li et al 1992; Klus et al 1996). We have reported that substituted 1-[(benzofuran-2-yl)phenylmethyl]imidazoles (**5**) are potent inhibitors of human testicular and bovine adrenal microsomal P450 17 (Al-Hamrouni et al 1997). Here we describe derivatives of these compounds (**1–4**) lacking the phenyl group (Owen 1995) as inhibitors of P450 17, and their in-vitro potency and specificity compared with ketoconazole with regard to other steroidogenic and liver enzymes.

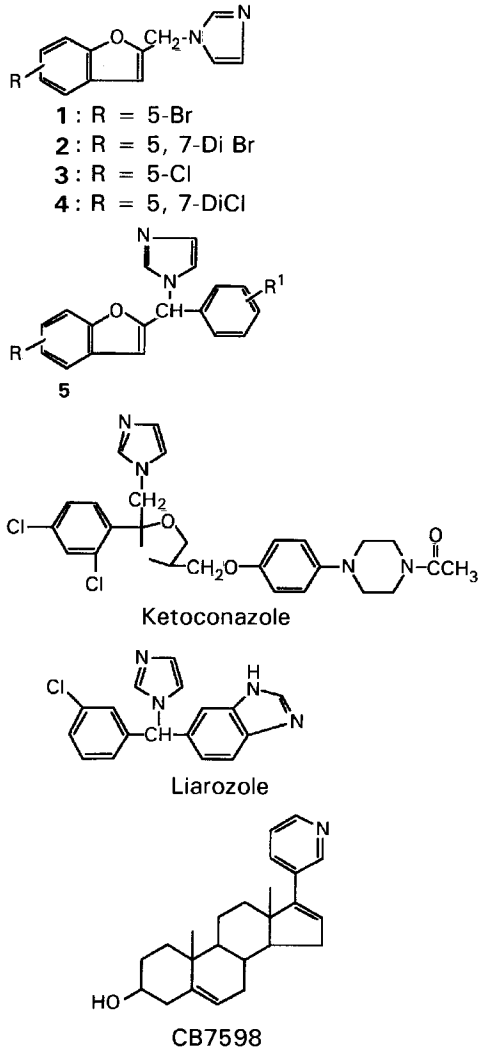


Figure 1. The structures of some P450 17 inhibitors.

Materials and Methods

Materials

The imidazoles (**1–4**) were synthesized in our laboratories (Owen 1995). [26,27-^{3}H]25-Hydroxycholesterol (84·5 Ci mmol^{-1}), [4-^{14}C]progesterone (60 mCi mmol^{-1}), [7-^{3}H]pregnenolone (25 Ci mmol^{-1}) and [1,2,6,7-^{3}H]progesterone (48·9 mCi mmol^{-1}) were obtained from NEN–DuPont (Stevenage, Herts, UK) and [1,2,6,7-^{3}H]androst-4-ene-3,17-dione (98 Ci mmol^{-1}) and 17α-hydroxy-[1,2,6,7-^{3}H]progeste-rone (65 Ci mmol^{-1}) from Amersham Life Science (Bucks, UK). All unlabelled steroids, sodium phenobarbital, formaldehyde, benzphetamine, D-glucose-6-phosphate (monosodium salt), NADP (monosodium salt) aluminium oxide 60G neutral (Type E), alumina N, and protein standards were obtained from Sigma (Poole, Dorset, UK). D-Glucose-6-phosphate dehydrogenase (grade R from yeast) was purchased from Boehringer Mannheim (Germany). Ketoconazole was a gift from Janssen Research (Belgium). Spectroscopic grade dimethyl-sulphoxide (DMSO) and all other reagents were obtained from BDH (Poole, Dorset). Thin-layer chromatography (TLC) plates were purchased from Whatman (Maidstone, Kent, UK).

NADPH-generating system consisted of glucose-6-phosphate (28·2 mg, 0·11 mmol), NADP (8·6 mg, 0·0116 mmol) and glucose-6-phosphate dehydrogenase (15 μL, 10 int. units) in phosphate buffer (pH 7·4, 50 mM, 1 mL).

Centrifugation was performed with an MSE Europa 65 M Ultracentrifuge (3000–110 000 *g*). Suspension of the microsomal fraction was

achieved with a Teflon pestle in a glass homogenizer (Potter–Elvejhem). Protein concentration was determined with a BCA Protein Assay Reagent obtained from Pierce (IL). Radioactivity was measured by use of an LKB Wallac 1217 Rackbeta Liquid Scintillation Counter. The scintillation fluids used were Instagel (Pharmacia Company, IL) and Optiphase Hisafe III (FSA Laboratory Supplies, Loughborough, Leics, UK). Ultraviolet absorption was measured by means of a Shimadzu UV-3000 spectrophotometer.

Enzyme preparation

Human testicular microsomal P450 17 and bovine adrenal microsomal P450 17 were prepared essentially by the methods of Al-Hamrouni et al (1997).

Bovine adrenal mitochondrial $P450_{SCC}$ and $P450_{11\beta}$. Eight bovine adrenal glands obtained from the local abattoir were transported to the laboratory on ice and processed at 4°C. The glands were defatted and demedullated and the cortex and connective tissues were removed from the capsule. The tissue was then weighed and homogenized in phosphate buffer. The homogenate was centrifuged at 900 *g* (1000 rev min^{-1}) for 20 min at 4°C, the pellet was discarded, and the supernatant was centrifuged at 10 000 *g* (12 000 rev min^{-1}) for 20 min at 4°C. The supernatant obtained was discarded and the pellet containing the mitochondria was suspended in phosphate buffer to give a protein concentration of 10–20 mg mL^{-1}. Samples (0·5 mL) of this suspension were transferred into capped plastic 1·5-mL tubes, snap frozen in liquid nitrogen and stored at −80°C until required.

Rat adrenal 10 000 g fraction $P450_{C21}$. Adrenal glands were removed from 16 male rats, 15–17 weeks, average weight 300 g, defatted, and homogenized in 50 mM phosphate buffer pH 7·4 (four times their weight) at 4°C with a Potter–Elvejhem homogenizer. The homogenate was then centrifuged at 10 000 *g* (12 000 rev min^{-1}) for 20 min at 4°C. The resulting supernatant was transferred to capped plastic tubes (1·5 mL), snap frozen in liquid nitrogen, and stored at −80°C until required.

Rat hepatic microsomal benzphetamine N*-demethylase.* Five male Wistar rats, 250–300 g, were given sodium phenobarbital (0·1% w/v) in their drinking water for five days to induce hepatic enzymes. They were then given phenobarbital-free water, fasted overnight and killed by stunning. The livers were removed, weighed, finely chopped with scissors and homogenized at 4°C in four times their weight of a solution containing 0·1 M Tris buffer (pH 7·4) and 0·25 M sucrose, by use of a Teflon homogenizer. The homogenate was centrifuged at 3000 rev min^{-1} (2000 *g*) for 15 min, and the supernatant retained and centrifuged for 20 min at 12 000 rev min^{-1} (10 000 *g*). The resultant supernatant was then centrifuged for 1 h at 36 000 rev min^{-1} (100 000 *g*), the supernatant being discarded and the pellet resuspended in Tris buffer (pH 7·4, 10 mL). This suspension was transferred to several plastic (1·5-mL) vials, frozen in liquid nitrogen and stored at −80°C until required for assay.

Rat testicular microsomal 17β-hydroxysteroid dehydrogenase. The testes were removed from 20 male Wistar rats, 12–15 weeks, average weight 300 g, after killing by stunning and exsanguination, and processed as described above for human testicular microsomal P450 17.

Enzyme assay

P450 17 human testicular microsomal enzyme was assayed essentially by the method of Al-Hamrouni et al (1997) using as the substrate 17α-hydroxy [1,2,6,7-^{3}H]progesterone and the unlabelled compound. Bovine adrenal microsomal enzyme was assayed essentially by the method of Al-Hamrouni et al (1997) using [7-^{3}H]pregnenolone (10 μL, 1 μM final concentration). Percentage conversion was given by 100 × disintegrations min^{-1} metabolites (17α-OH pregnenolone + androstenediol + dehydroepiandrosterone) / total disintegrations min^{-1}.

Assay of $P450_{SCC}$. The assay mixture (1 mL) consisted of phosphate buffer (pH 7·4, 775 μL), [26,27-^{3}H]25-hydroxycholesterol and unlabelled compound in ethanol containing Tween 80 as substrate (100 μL, 24·81 μM), NADPH generating system (50 μL). The reaction was started by addition of sonicated enzyme (0·7 mg protein mL^{-1}, 75 μL) and after incubation at 37°C for 30 min the reaction was terminated by pipetting a sample (500 μL) into sodium hydroxide (100 μL, 0·1 M) and mixing thoroughly. Unmetabolized substrate was removed by extracting the mixture with chloroform (1 mL), the tubes were centrifuged for 10 min at 1500 rev min^{-1} (950 *g*) and samples (500 μL) of the supernatant were added to tubes containing 50% alumina mixture. These tubes were then centrifuged for 15 min at 3000 rev min^{-1} (2000 *g*). Samples (500 μL) were then dispersed into scintillation fluid (Optiphase Hisafe) (2 mL) and counted (^{3}H) for 5 min by means of an LKB Wallac liquid scintillation counter. The assay was linear up to a protein concentration of 2·8 mg mL^{-1} and a time period of 40 min.

Assay of P450$_{C21}$. [4-^{14}C]Progesterone (10 μL, 2·12 μM final concentration) in propylene glycol was mixed with 50 mM phosphate buffer (pH 7·4, 410 μL) and rat adrenal microsomes (30 μL, 1·38 mg protein mL^{-1}). The reaction was initiated by the addition of NADH generating system (50 μL) and the mixture incubated at 37°C for 10 min. The reaction was terminated by addition of diethyl ether (3 mL) and the steroids extracted with diethyl ether (2 × 3 mL). The solvent was evaporated and the extract reconstituted in acetone (50 μL) and analysed by TLC with chloroform–ethyl acetate, 4:1, as mobile phase. Unlabelled progesterone and 21-hydroxyprogesterone were also chromatographed on the same plate for identification of the spots. From this point the experimental procedure was identical with that described for assay of P450 17. The assay was linear up to a protein concentration of 1·84 mg mL^{-1} and a time period of 15 min.

Assay of P450$_{11\beta}$. 21α-Hydroxy[1,2,6,7-^{3}H]progesterone (10 μL, final concentration 1 μM), prepared from labelled progesterone (Bahshwan 1997), in propylene glycol was incubated at 37°C with NADPH-generating system (50 μL) and phosphate buffer (pH 7·4, 910 μL) and the reaction started by addition of sonicated enzyme (30 μL, 0·21 mg mL^{-1}). The reaction was terminated after 15 min by addition of diethyl ether (3 mL) and the steroids were extracted with diethyl ether (2 × 3 mL). The solvent was evaporated and the extract reconstituted in acetone (50 μL) and analysed by TLC with chloroform–ethyl acetate–methanol, 8·5:2·5:0·5, as mobile phase. Location of labelled steroids (deoxycorticosterone and corticosterone) on TLC plates was aided by chromatographing unlabelled steroids on the same plate.

Spots corresponding to corticosterone and deoxycorticosterone were scraped from the plates and transferred to counting vials containing 50% aqueous methanol (1 mL) and scintillation fluid (2 mL Hisafe Optiphase III), mixed thoroughly, and counted on a liquid scintillation counter. Percentage conversion was given by 100 × [(disintegrations min^{-1} corticosterone)/(disintegrations min^{-1} deoxycorticosterone + corticosterone)]. The assay was linear up to a protein concentration of 0·21 mg mL^{-1} and a time period of 15 min.

Assay of benzphetamine N-*demethylase.* The procedure used was based essentially on that of Gibson & Skett (1994). Benzphetamine (0·5 mL, 1·25 mM final concentration), NADPH-generating system (0·5 mL) and 50 mM phosphate buffer, pH 7·4 (0·48 mL) were incubated at 37°C and the reaction initiated by addition of the rat-liver microsomal suspension (0·5 mL, 2·87 mg mL^{-1} final concentration). The reaction was terminated after 30 min by addition of 25% zinc sulphate solution (0·5 mL). In blank incubations the substrate was added just before the termination step. After addition of saturated barium hydroxide (0·25 mL), the tubes were vortex-mixed and centrifuged at 2000 *g* (3000 rev min^{-1}) for 10 min. Samples (0·8 mL) of the resulting supernatant and formaldehyde standards (0·2–1 μg mL^{-1}) were added to Nash reagent (2 mL) and the tubes were incubated at 60°C in a water bath for 30 min. The tubes were then left to cool to room temperature and the samples were transferred to plastic cuvettes and the spectrophotometric absorbance measured at 415 nm. The formaldehyde liberated during *N*-demethylation of benzphetamine was calculated by reference to a standard curve. A small amount of endogenous formaldehyde formed by the blanks was subtracted from the total amount of formaldehyde formed in the sample tubes.

Assay of 17β-hydroxysteroid dehydrogenase. The substrate [1,2,6,7-^{3}H]androst-4-ene-3,17-dione and unlabelled substrate (10 μL, 10 μM final concentration) in propylene glycol, 50 mM phosphate buffer pH 7·4 (420 μL) and NADPH-generating system (50 μL) were incubated at 37°C and the reaction initiated by addition of the rat testicular microsomal suspension (20 μL, 0·124 mg mL^{-1} final concentration). The reaction was terminated after 30 min by addition of diethyl ether (3 mL) and the steroids were extracted with diethyl ether (2 × 3 mL). The solvent was evaporated and the extract reconstituted in acetone (50 μL) and analysed by TLC with chloroform–ethyl acetate–methanol (85:12·5:2·5) as mobile phase. From this point the experimental procedure was identical with that described for assay of P450 17. The assay was linear up to a protein concentration of 0·186 mg mL^{-1} and a time period of 40 min.

Inhibition

Inhibitors (100 μM, 10 μL) in ethanol were added to the assay mixtures. In control incubations ethanol (10 μL) was substituted for the inhibitor solution. % Inhibition = [(% conversion (or activity) of control − % conversion (or activity) of inhibitor)/(% conversion (or activity) control)] × 100. The results in Table 2 are the means of duplicate determinations; the spread was < 5%. IC50 values were determined by use of a range of inhibitor concentrations in ethanol (10 μL) and a single substrate concentration. In control

incubations ethanol (10 μL) was substituted for the inhibitor solution. The IC50 value was determined graphically from a plot of (% activity remaining) against Log [I] using Cricket Graph. The means are for triplicate determinations and R^2 was in the range 0·938–0·990 ($P450_{11\beta}$), 0·980–0·988 ($P450_{C21}$) or 0·981–0·995 (17β-hydroxysteroid dehydrogenase).

Spectral studies

Qualitative studies. Equal volumes (2·5 mL) of liver microsomes (2 mg protein mL^{-1}) in Tris buffer (0·1 M, pH 7·4) were added to both sample and reference-matched cuvettes and the spectrophotometric baseline was recorded from 500–350 nm. A sample (10 μL) of the test compounds dissolved in DMSO was added to the microsomal suspension to give a final concentration of 0·4 mM. A similar volume of DMSO was added to the reference cuvette and the differential absorption spectrum was recorded. The compounds under test did not absorb in the 500–350 nm region.

Quantitative studies. These were conducted as described above except that samples (10 μL, 0·4 mM) of the test compound solution were added consecutively. After standing for 10 s on each occasion the differential absorption spectrum was acquired. A plot of $1/\Delta A$ against 1/[inhibitor] using Cricket Graph gave a straight line which intersected the x axis at $-1/K_s$. The means are for duplicate determinations and R^2 was in the range 0·884–0·987.

Results and Discussion

All four compounds were inhibitors of human testicular microsomal P450 17 but were less potent (IC50 = 0·18–0·38 μM) than ketoconazole (0·03 μM); **4**, the most potent, was six-fold less potent than ketoconazole (Table 1). Previous work (Al-Hamrouni et al 1997) has shown that in the corresponding series of phenyl substituted compounds (1-[(benzofuran-2-yl)phenylmethyl]imidazoles, **5**) the 5-chloro compound was 15-fold less potent than ketoconazole but an additional 2′-chloro substituent (phenyl ring) increased potency to half that of ketoconazole. The potency of unsubstituted benzofuran ring compounds was approximately equal to that of ketoconazole for the 2′-OCH_3, 2′-CH_3, 4′-Cl and 4′-F phenyl-substituted compounds. It would seem that the absence of a suitably substituted phenyl group in the novel compounds described here accounts for the reduced potency.

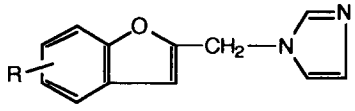
The adrenal P450 17 enzyme in man accounts for a small (5–10%) but important proportion of the body's production of testosterone through the peripheral metabolism of adrenal androstenedione and dehydroepiandrosterone. Ketoconazole is a potent inhibitor of the adrenal and testicular enzyme whereas the more recently developed liarozole has less effect on the adrenal enzyme (Vanden Bossche & Willemsens 1991). The novel compounds were compared with ketoconazole for their inhibitory effects on the readily available bovine adrenal microsomal P450 17. All four compounds (IC50 = 1·15–1·8 μM) were 22–35-fold more effective inhibitors than ketoconazole (39·8 μM) (Table 1). Similarly enhanced inhibitory activity was noted (Al-Hamrouni et al 1997) for the phenyl-substituted compounds, **5**, (see above) where the 5-chloro compound had approximately 500-fold greater activity but substitution in the phenyl ring reduced the activity to 4–40-fold that of ketoconazole.

Cholesterol side-chain cleavage enzyme ($P450_{SCC}$) catalyses the conversion of cholesterol to pregnenolone in the steroidogenic pathway. Inhibition of this enzyme leads to depletion of corticosteroids (cortisol) and release of adrenocorticotrophic hormone (ACTH) from the pituitary to stimulate the pathway. Consequently inhibition of this enzyme by a potential reversible inhibitor targeted at P450 17 could increase progesterone and pregnenolone levels in the adrenal gland which could overcome or reduce the inhibition of P450 17. The aromatase inhibitor, aminoglutethimide (AG), also inhibits $P450_{SCC}$, albeit with a lower potency (one tenth) than that toward aromatase (Shaw et al 1988), so that its clinical use necessitates adjuvant therapy with hydrocortisone to maintain levels of this hormone and prevent feed-back stimulation of the pathway through ACTH release.

The screening of the four compounds as inhibitors of $P450_{SCC}$ shows that they are less potent (10·2–31·7% inhibition; Table 2) than AG (67%) or ketoconazole (95·9%). The most potent of the novel compounds was **3**; its inhibitory activity was approximately half that of AG and one third that of ketoconazole. However, the inhibition (95·9%) observed with ketoconazole was at the upper limits of accurate detection of potency by the assay which would, therefore, be expected, from our experience, to underestimate the potency of ketoconazole relative to those of the other compounds studied.

$P450_{C21}$ catalyses the conversion of progesterone and 17α-hydroxyprogesterone to deoxycorticosterone and 11-deoxycortisol, respectively. All the compounds were more potent inhibitors (inhibition 21–62·1%) of the enzyme than ketoconazole (7·9%) (Table 2), which indicates that an increase in the in-vivo dose to offset the lower potency of these

Table 1. Inhibition of P450 17 from human testes and bovine adrenal microsomes by some substituted 1-[(benzofuran-2-yl)methyl] imidazoles and by ketoconazole.



Compound	R	Inhibition (IC50 (μM) ± s.d.)	
		P450 17 from human testicles*	P450 17 from bovine adrenal glands†
1	5-Br	0·380 ± 0·04	1·54 ± 0·06
2	5,7-DiBr	0·185 ± 0·021	1·15 ± 0·003
3	5-Cl	0·230 ± 0·043	1·80 ± 0·07
4	5,7-DiCl	0·180 ± 0·033	1·30 ± 0·025
Ketoconazole	–	0·029 ± 0·003	39·80 ± 0·13

*17α-Hydroxyprogesterone, 6 μM. †Pregnenolone, 1 μM. Values are the means ± s.d. of results from three determinations, each in triplicate.

compounds towards P450 17 would have much greater effects on deoxycorticosterone and 11-deoxycortisol than ketoconazole.

11β-Hydroxylase ($P450_{11\beta}$) is an adrenal enzyme also, like $P450_{C21}$, involved in corticosteroid production. In man 11β-hydroxylase converts deoxycorticosterone and 11-deoxycortisol to corticosterone and cortisol, respectively. The mineralocorticoid aldosterone is also produced by this enzyme through 18-hydroxylation of cortisol and it would seem that the adrenal gland in man contains two forms of the enzyme only one of which can synthesize aldosterone (Oritz de Montellano 1986). The same is true of the bovine and rat adrenal enzymes (De Coster et al 1989). Interference with aldosterone production and consequently Na^+ retention, or cortisol production by an agent targeted at P450 17 would have a serious side effect. When the aromatase inhibitor fadrazole was used clinically it was shown at high doses to inhibit 18-steroid hydroxylation; this led to the introduction of letrozole which is much more selective to aromatase (Bhatnager et al 1990).

The standard method of assessing the in-vitro selectivity of inhibitors of a target enzyme compared with ketoconazole has so far been used here. However a novel means of obtaining a quantitative selectivity ratio compared with ketoconazole is to use a ratio of IC50 values, where these are available, as set out in formula 1. A value > 1 indicates less selectivity than that of ketoconazole to P450 17 and a value < 1 indicates greater selectivity.

$$\begin{aligned}&(\text{P450 17 IC50 compound/}\\&\text{P450 17 IC50 ketoconazole})\\&\times (P450_{11\beta}\ \text{IC50 ketoconazole/}\\&P450_{11\beta}\ \text{IC50compound})\end{aligned} \quad (1)$$

Here, using the readily available bovine adrenal gland mitochondrial enzyme ($P450_{11\beta}$), three of the novel compounds were found to be more potent (IC50 0·5–81 μM) than ketoconazole (90·9 μM) but **2** was less potent (138·4 μM) (Table 2). By use of formula 1, the in-vivo selectivity of **1**, **2**, **3** and **4** compared with ketoconazole was calculated as

Table 2. Inhibition profiles of some substituted 1-[(benzofuran-2-yl)methyl]imidazoles and ketoconazole.

	Compound				
	1	**2**	**3**	**4**	Ketoconazole
Inhibition (%) of $P450_{scc}$ (100 μM)*	21·9	13·8	31·7	10·2	95·9
Inhibition (%) of $P450_{c21}$ (100 μM)†	32·5	57	21	57·7	7·9
$P450_{11\beta}$ IC50‡ (μM)§	0·5	138·4	0·5	81	90·9
Inhibition (%) of benzphetamine oxidation (100 μM)¶	51·4	38·7	52·7	47·3	15·0
Inhibition (%) of benzphetamine oxidation (200 μM)¶	59·5	43·7	59·8	54·0	39·5
Binding to hepatic microsomes, K_s (mM)	0·35	0·12	0·06	0·11	0·72
Inhibition (%) of 17β-hydroxysteroid dehydrogenase (200 μM)**	22·2	85·5	14·9	46·5	69

*Substrate 25-hydroxycholesterol, 24·81 μM. † Substrate progesterone, 2·12 μM. ‡Concentration resulting in 50% inhibition. §Substrate 21α-hydroxyprogesterone, 1 μM. ¶Substrate benzphetamine, 1·25 mM. **Substrate androstenedione, 10 μM.

2320, 4·16, 1380, and 6·72, respectively. Compounds **2** and **4** have less in-vivo selectivity than ketoconazole, although of the same order, and it is likely that corticosteroid and aldosterone production would be affected to a greater extent.

17β-Hydroxysteroid dehydrogenase is an ubiquitous enzyme in man, occurring in the endometrium, placenta, liver, prostate, testes, breast, skin and adipose tissue (Martel et al 1992). Several pure isoforms, designated Types 1–6 have been isolated (Andersson 1995; Biswas & Russell 1997). The interconversion oestrone ↔ oestradiol is catalysed in the forward direction by Type 1 isoenzyme and in the reverse direction by Type 2. Androstenedione is the major substrate for human testicular Type 3 isoenzyme, leading to testosterone production. Testosterone is the precursor of 5α-dihydrotestosterone responsible for driving prostatic cancer growth. A P450 17 inhibitor which also targeted the testicular 17β-hydroxysteroid dehydrogenase enzyme would have the useful side effects of not only reducing androstenedione production but also preventing any breakthrough of this weak androgen being converted by 17β-hydroxysteroid dehydro-genase to the potent androgen testosterone.

In this work the rat testicular microsomal enzyme was used because of the non-availability of the enzyme from man and it must be considered that it might not correspond to the human type 3 enzyme because a correlation has not yet been reported.

Screening of **1–4** and ketoconazole at 200 μM (Table 1) in the reductive step showed that only **2** (85·5% inhibition), ketoconazole (69%) and **4** (46·5%) had appreciable inhibitory potency towards the enzyme (Table 2).

The IC50 values (not shown) for **2** and for ketoconazole were 75·4 ± 0·05 and 104·1 ± 0·08 μM respectively which, using formula 1, gave a selectivity ratio of 8·8 indicating that **2** would have a greater effect than ketoconazole on the in-vivo conversion of androstenedione to testosterone, although the effects of both inhibitors would be weak.

The effect of the four novel compounds and ketoconazole on cytochrome P450 enzymes responsible for metabolism of xenobiotics was examined to determine their potential interference with the metabolism of other drugs given concurrently in the event of their use as potential P450 17 inhibitors. The interference with the metabolism of benzphetamine through *N*-demethylation by rat liver microsomes in-vitro was used as a well established model (Gibson & Skett 1994). Ketoconazole has been reported to be hepatotoxic (Lake-Bakaar et al 1987) but this could be unrelated to the model used here.

At a concentration of 100 μM (Table 2) all the compounds inhibited the enzyme(s) (38·7–52·7%) and were more potent than ketoconazole (15%). The moderate effects were not concentration-dependent and levelled out as shown when higher concentrations of 200 μM and 400 μM (not shown) were used. These figures show that all the compounds are less selective towards P450 17 than is ketoconazole and that effects on hepatic enzymes would be greater than for ketoconazole.

Binding of the novel compounds and ketoconazole to rat liver microsomes was studied by difference spectroscopy. Spectral changes characteristic of type II spectra (λ_{max} 420–435 nm and λ_{min} 390–410 nm) were observed indicative of binding of the imidazole-nitrogen lone pair to the iron-haem. Quantitative determination of the strength of the binding (K_s) of the compounds to the cytochrome(s) showed that all the compounds were bound more strongly ($K_s = 0{\cdot}06$–$0{\cdot}53$ μM) than ketoconazole (0·72 μM) (Table 2).

The binding, as measured by K_s, reflects overall binding to cytochrome P450s in the liver microsomes and is not restricted to benzphetamine *N*-demethylase of concern in this work. However, it was considered of interest to attempt to correlate the noted K_s values with inhibitory potencies towards the other enzymes examined here although it seemed unlikely that such correlation would be found in-view of the different binding sites (other than the Fe-haem) occurring in these targetted enzymes.

Work by Pepper et al (1994) in these laboratories with placental microsomes aromatase showed that inhibitory potency within a series of closely related 3-(4-aminophenyl)pyrrolidine-2,5-diones followed a qualitative but not quantitative pattern. Examination of the correlation plots (not shown) for either percent inhibition or IC50 values of the compounds and ketoconazole for the enzyme targets studied here against the K_s values for their hepatic microsomal P450 binding showed no correlation between inhibitory potency and K_s.

Conclusions

Compound **2** is a useful lead compound for development as a P450 17 inhibitor for the treatment of prostate cancer, although six-fold less active than ketoconazole towards human testicular microsomal P450 17. The in-vitro selectivity of **2** compared with ketoconazole could indicate equivalent selectivity for $P450_{SCC}$ at the enhanced dose required for equivalent effects on P450 17 in-vivo, but less selectivity for enzymes affecting

cortisol and aldosterone production, $P450_{11\beta}$ and $P450_{C21}$, and greater interaction with hepatic P450.

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