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R 76713 AND ENANTIOMERS: SELECTIVE, NONSTEROIDAL INHIBITORS OF THE CYTOCHROME P450-DEPENDENT OESTROGEN SYNTHESIS

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Abstract—The triazole derivative, R 76713 and its enantiomers R 83839(−) and R 83842(+) are effective inhibitors of the aromatization of androstenedione. For human placental microsomes, the (+) enantiomer (R 83824) is about 1.9- and 32-times more active than the racemate (IC_{50} 2.6 nM) and the (−) enantiomer, respectively. R 83842 is about 30- and 1029-times more active than 4-hydroxyandrostene-3,17-dione and aminoglutethimide. This potency might originate from its high affinity for the microsomal cytochrome P450 (P450). Indeed, R 83842, compared to R 76713 and R 83839, forms a more stable P450–drug complex. Difference spectral measurements indicate that the triazole nitrogen N-4 coordinates to the haem iron. The reversed type I spectral changes suggest that R 76713 is able to displace the substrate from its binding place and the stable complex formed in particular with the (+) enantiomer suggests that its N-1-substituent occupies a lipophilic region of the apoprotein moiety. Kinetic analysis implies that there is a competitive part in the inhibition of the human placental aromatase by R 76713. The K_i values for R 76713, R 83842 and R 83839 are 1.3 nM, 0.7 nM and 18 nM, respectively. These results are indicative of stereospecificity for binding. Up to 10 μM, R 76713 and its enantiomers have no statistically significant effect on the regio- and stereoselective oxidations of testosterone in male rat liver microsomes. All three compounds have no effect on the P450-dependent cholesterol synthesis, cholesterol side-chain cleavage and 7α-hydroxylation and 21-hydroxylase. At 10 μM, R 76713 has a slight effect on the bovine adrenal 11β-hydroxylase. This effect originates mainly from R 83839, the less potent aromatase inhibitor. On the other hand, the inhibition of the 17,20-lyase of rat testis observed at concentrations $\geq$0.5 μM, originates rather from R 83842. However, 50% inhibition is only achieved at 1.8 μM R 83842, i.e. at a concentration about 1300-times higher than that needed to reach 50% inhibition of the human placental aromatase.

The cytochrome P450 (P450)-dependent aromatization of androgens into oestrogens is one of the most important reactions involved in steroid hormone biosynthesis. Androgens (androstenedione, testosterone, 16α-hydroxytestosterone) are converted into oestrogens by two hydroxylations at the C-19 methyl group and a third one at C-2. This results in the loss of the C-19 methyl group and aromatization of the steroid A ring [1, 2]. The three successive oxidation reactions are catalysed by one single P450, $P450_{AROM}$ (protein name: XIXA1 [31]) [4–6].

The aromatase enzyme complex is present in the granulosa cells of the ovaries [7], in adipose tissue of both males and females [8–10], skin fibroblasts [11, 12], the Sertoli cells of the testis [2], several areas of the brain [13], placenta [14–16], rat prostate and Dunning R3327H rat prostatic adenocarcinoma [17], and human neoplastic endometrium [18] and breast tumor [10, 19, 20]. Assays of aromatase in adipose tissue from different quadrants of mastectomy specimens from patients with breast cancer indicate that the activity is always higher in quadrants associated with tumor as compared with non-involved quadrants [21].

Patients having oestrogen receptors in their breast tumor biopsies respond to deprivation of oestrogens [22]. Thus, an oestrogen biosynthesis inhibitor might be of help in the treatment of oestrogen-dependent disorders such as breast cancer and also gynaecomastia and endometriosis [23]. The aromatse inhibitor aminoglutethimide has been found to inhibit oestrogen production in postmenopausal breast cancer patients and to produce objective disease remission [22]. However, aminoglutethimide is a non-specific inhibitor, and blocks beside the aromatase, the P450-dependent cholesterol side-chain cleavage, 11β-hydroxylase [22], 21-hydroxylase and 18-hydroxylase [23].

The present study was designed to examine the interaction of R 76713 (Fig. 1) and its enantiomers, R 83839(−) and R 83842(+) with the human placental microsomal aromatase and other microsomal and mitochondrial P450 isozymes and P450-dependent reactions. The results obtained show that R 76713 has a high affinity for microsomal P450 from human placenta and is a potent and selective inhibitor of the human placental aromatase. The aromatase inhibitory effect of R 76713 is largely due to its (+) enantiomer, R 83842.

MATERIALS AND METHODS

Membrane fractions

The isolation of mitochondria or microsomes from

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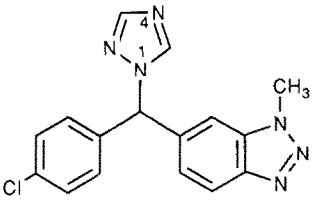

R 76713 (±); R 83839 (-); R 83842 (+)

Fig. 1. Chemical structure of R 76713.

bovine adrenal cortex [24], piglet [25] and rat testes [26], and of S-10,000 fractions (i.e. the supernatant of a 10,000 *g* centrifugation) [26] was as described previously.

Male, New Zealand white rabbits (2.5 kg) were injected intraperitoneally with 3-methylcholanthrene (3-MC) dissolved in olive oil, or with phenobarbital, dissolved in water, for 8 days at a daily dose of 25 mg/kg or 80 mg/kg body weight, respectively. Rabbits were killed 24 hr after the last dose. Livers and lungs were removed, minced with a pair of scissors and washed with 4 L of cold NaCl 0.9%. The minced livers were homogenized in 4 vol. (w/v) and the lung tissue in 2 vol. (w/v) of 1.15% KCl using a Potter–Elvehjem homogenizer with a Teflon pestle. The homogenate was centrifuged twice at 1500 *g* for 10 min and the cell-free supernatant at 10,000 *g* for 20 min. The supernatant was centrifuged as before and the final supernatant centrifuged at 105,000 *g* for 60 min. The pellet was resuspended in 1.15% KCl and centrifuged as before. The final pellet was suspended in 0.1 M potassium phosphate buffer (pH 7.4) and stored at −80°. Microsomes from untreated rabbits were obtained following the same procedure.

Placentas were obtained after full-term deliveries from a local hospital. They were collected in ice-cold NaCl 0.9% and brought to the laboratory within 30 min. Large vessels, fat and membranes were removed, the tissue was washed at 4° with 4 L of NaCl 0.9% and minced with a pair of scissors. The minced tissue was homogenized in 3 vol. (w/v) of 0.25 M sucrose (containing 10 mM sodium phosphate and 0.1 mM EDTA; pH 7.4) using a Polytron® homogenizer, equipped with a PTA 20 S generator. The homogenate was centrifuged for 10 min at 800 *g* and the supernatant was centrifuged at 15,000 *g* for 20 min. The supernatant was retained and recentrifuged at 140,000 *g* for 30 min. The microsomal pellet was resuspended in 50 mM sodium phosphate buffer containing 100 mM KCl, 1 mM EDTA, 1 mM dithiothreitol and 10% glycerol, pH 7.4, and recentrifuged at 140,000 *g* for 30 min. The final pellet was resuspended in the above described buffer at a protein content of about 30 mg/mL [27], and stored at −70°. For the kinetic studies the pellet of the first 140,000 *g* centrifugation was resuspended in 100 mM sodium pyrophosphate buffer (pH 7.4) containing 5 μg/mL butylated hydroxytoluene (BHT) and 2 μM androstenedione and recentrifuged at 140,000 *g* for 45 min. The washed microsomal pellet was resuspended in 50 mM sodium phosphate buffer (pH 7.4) containing 100 mM KCl, 1 mM EDTA, 2 μM androstenedione, 5 μg/mL BHT, 1 mM dithiotreitol and 20% glycerol, and stored at −70°.

Spectrophotometric analysis

The P450 content of the subcellular fractions was determined according to Omura and Sato [28] by measuring the reduced carbon monoxide difference spectrum (extinction coefficient used was 91 cm^{-1} mM^{-1}). The membrane fractions were diluted in 0.1 mM potassium phosphate buffer (pH 7.4) to obtain a P450 content of 0.1 nmol/mL. The suspension was divided between the reference and sample cuvettes, drugs and/or dimethylsulfoxide (DMSO) were added to both cuvettes and the P450s were reduced with a few grains of sodium dithionite. The sample cuvette was bubbled for 30 sec with carbon monoxide and then tightly closed. The difference spectrum was recorded 45 sec later.

To trace the spectral transitions of the Soret band of P450 associated with the addition of substrate or drugs, the microsomal suspension was divided between the sample and reference cuvettes. A baseline of equal light absorbance was established, substrate or drug was added to the sample and solvent to the reference cuvette and the resulting difference spectrum was recorded. In some experiments equal amounts of drug or solvent were added afterwards to the reference and sample cuvette, respectively. After reduction of the P450s present, CO was bubbled through the sample cuvette and the difference spectra recorded in function of time.

Aromatase

The conversion of androstenedione to oestrone was assayed according to Kellis and Vickery [27]. In summary, the incubation volume (1 mL) contained 50 mM sodium phosphate (pH 7.2), 40 nM [4-^{14}C]androstenedione (sp. act. 52 mCi/mmol), 10 μL of drug and/or ethanol, 25 μg microsomal protein, 2.5 mM glucose-6-phosphate and 0.25 units glucose-6-phosphate dehydrogenase. The mixture was preincubated for 25 sec at 37°. The reaction was started adding 0.1 mM NADPH and stopped after 6 min at 37° by the addition of 7 mL of ethylacetate [29]. The mixture was agitated on a Vortex® mixer and centrifuged at 600 *g* for 5 min. The aqueous phase was re-extracted with 7 mL of ethylacetate and the combined extracts were evaporated to dryness under a stream of nitrogen. The residues were dissolved in 50 μL of a solvent mixture containing chloroform: methanol:ethylacetate (2:1:3, by vol. and 2 mg/mL of testosterone, androstenedione, oestrone and oestradiol. Steroids were separated on precoated silicagel 60 F254 aluminium plates (Merck, Darmstadt, F.R.G.) using ethylacetate:isooctane (70:30, v/v). After separation, the steroids were localized and the radioactivity was determined as described previously [30].

Cholesterol and steroid synthesis

Cholesterol synthesis from [^{14}C]mevalonate by rat liver subcellular fractions (S-10,000) was studied by the method of Mitropoulos *et al.* [31]. The incubation mixture contained in a final volume of 5 mL, 4 mL of the S-10,000 fraction (15 mg protein/mL), 16 mM

fructose-1,6-diphosphate, 1.6 mM NAD, 4 mM $MgCl_2 \cdot 6H_2O$, 10 μL drug and/or solvent and 1.25 μCi [^{14}C]mevalonate (sp. act. 51 mCi/mmol). Incubations were carried out in a reciprocating shaker at 37° for 3 hr. The incubation mixtures were gassed with 0.85 L of air per min. After incubation, the lipids were saponified, extracted with *n*-heptane and separated by TLC using heptane/diisopropyl ether/acetic acid (60:40:4, by vol.) as solvent system. Cholesterol side-chain cleavage was studied in extracts from sonicated bovine adrenal cortex mitochondria as described previously [24]. Incubations were carried out at 37° for 1 hr in a final volume of 2.5 mL containing 1.5 mL mitochondrial extract (8 mg protein/mL), 1 mL 0.1 mM potassium phosphate buffer (pH 7.4), 25 μmol $MgSO_4 \cdot 7H_2O$, 2.4 μmol NADP, 20.5 μmol glucose-6-phosphate, 0.5 units of glucose-6-phosphate dehydrogenase, 10 μL drug and/or solvent and 0.2 μCi [4-^{14}C]cholesterol in 50 μL acetone. After incubation, the steroids were extracted on Extrelut-3® (Merck) with 15 mL diethyl ether and separated by TLC using cyclohexane/ethylacetate (60:40, v/v) as solvent system.

Cholesterol 7α-hydroxylation was measured in female rat (Wistar) liver microsomes prepared according to Princen *et al.* [32]. The standard incubation mixture [33] contained in a final volume of 5 mL, 3 mL of the microsomal fraction (2 mg protein/mL), 10 μL of drug and/or solvent, and a 0.1 mM potassium phosphate buffer (pH 7.4) containing 30 mM nicotinamide, 5 mM $MgCl_2 \cdot 6H_2O$, 1 mM NADP, 10 mM glucose-6-phosphate and 1 unit of glucose-6-phosphate dehydrogenase. The substrate [4-^{14}C]cholesterol (0.5 μCi, sp. act, 60 mCi/mmol) was solubilized in 0.1 mL methanol to which 3 mg Tween 80 was added. The methanol phase was evaporated under nitrogen and 0.1 mL of buffer was added [34]. Cholesterol was resuspended on a Vortex® mixture. After the substrate was added to the mixture, the incubations were carried out for 2 hr at 37° with constant shaking. The incubations were terminated by adding 30 mL of chloroform/methanol (2:1) containing 0.01% BHT and the chloroform layer evaporated under nitrogen. The products of incubation were applied to precoated silicagel 60 F254 aluminium plates, which were twice developed with toluene/ethylacetate (2:3). Products present were vizualized with autoradiography, and the radioactivity determined as described previously [30].

Androgen biosynthesis. [^{14}C]Pregnenolone metabolism by S-10,000 fractions of Wistar rat testes was studied by incubating 4 mL S-10 fraction (5 mg protein/mL), 0.97 mM NADP, 2.48 $MgCl_2 \cdot 6H_2O$, 2.48 mM ATP, 8.22 mM glucose-6-phosphate, 0.98 units of glucose-6-phosphate dehydrogenase, 5 μL R76713 and/or DMSO and 0.1 μCi [4-^{14}C]pregnenolone. The final volume was 5 mL. Incubations were carried out in 25-mL Erlenmeyer flasks at 37° in a reciprocating shaker. During the entire period, the incubation mixtures were gassed with air. At the end of the 30 min-incubation period, 2.5 mL were applied to Extrelut 3®-columns and after 10 min steroids were extracted with 15 mL diethylether. After evaporation of the solvent under a stream of nitrogen the steroids were separated by a two dimensional TLC-system on precoated silica gel 60 F_{254} plates using as first solvent system ethylacetate:dichloromethane (60:40) and as second system isoctane:diisopropylether:acetic acid:ethylacetate:toluene (200:125:175:200:300). Radioactivity present in the different steroids was determined as previously described [26].

[^{14}C]Pregnenolone incorporation into steroids of bovine adrenal cortex microsomes (androstenedione, dehydroepiandrosterone, 11-deoxycortisol and 11-deoxycorticosterone) was measured as described for the S-10,000 fractions of rat testes.

Effects on 17,20-lyase were studied using a S-10,000 fraction of rat testes and the same incubation circumstances as described for the studies of the androgen synthesis from pregnenolone. The substrate, [^{3}H]17α-hydroxy-20-dihydroprogesterone was prepared from [1, 2, 6, 7^{3}H]17-hydroxyprogesterone as described previously [26].

The incorporation of [^{3}H]11-deoxycortisol into cortisol (11β-hydroxylase activity) was measured using bovine adrenocortical mitochondria [24].

Testosterone metabolism. Male Sprague–Dawley rats (11 weeks old) were pretreated for 7 days with phenobarbital (1 g/L in the drinking water). Rats were killed, the livers were removed, minced in 0.9% NaCl, washed to remove blood and homogenized in 4 vol. (w/v) of 1.15% KCl using a Potter–Elvehjem homogenizer with Teflon pestle at 4°. The homogenate was centrifuged for 10 min at 1500 *g*. The supernatant was recentrifuged for 10 min at 1500 *g* and the supernatant thus obtained centrifuged twice for 20 min at 10,000 *g*. The final supernatant was centrifuged for 60 min at 105,000 *g*. The pellet, resuspended in KCl 1.15% was recentrifuged for 60 min at 105,000 *g* [24] and the final pellet resuspended in 0.1 M sodium phosphate buffer containing 20% glycerol (pH 7.4) and stored at −80°. To measure testosterone metabolism, the reaction mixture contained in a total volume of 1 mL: 0.8 mL microsomal suspension (protein content 0.3 mg/mL), 5 μL R 76713, R 83839 or R 83842 and/or DMSO, 5 mM $MgCl_2 \cdot 6H_2O$, 0.5 mM NADP, 8.22 mM glucose-6-phosphate, 0.98 units glucose-6-phosphate dehydrogenase and 0.02 M sodium phosphate buffer (pH 7.4) containing 5 μM $MnSO_4$. The reaction was started by the addition of 100 nmol testosterone of which 4 nmol was [4-^{14}C]testosterone. After 30 min of incubation at 37° in a Heto® shaking bath, the reaction was terminated by adding 4 mL ice-cold sodium phosphate buffer (0.1 M, pH 7.4) containing 5 μM $MnSO_4$ and 2.5 mL were applied on Extrelut 3® columns. After 10 min the steroids were extracted with 15 mL diethylether. After evaporation of the solvent under a stream of nitrogen, the steroids were dissolved in 60 μL ethylacetate and separated by TLC and/or HPLC.

TLC. The steroids were separated on precoated silica gel 60 F_{254} aluminium plates as described by Waxman *et al.* [35]. The plates were developed twice using dichloromethane:acetone (8:2, v/v) as solvent system. After separation, radioactive spots were localized using contact photography and radioactivity determined as described previously [26].

HPLC. HPLC analyses were carried out on a Varian 5560 Liquid Chromatograph equipped with

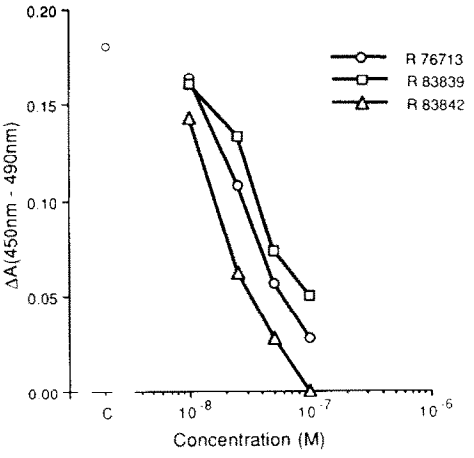

Fig. 2. Increasing concentrations of R 76713, R 83839, R 83842 and/or DMSO were added to suspensions of human placental microsomes containing P450 in the oxidized form. After reduction and addition of carbon monoxide to the sample cuvette, a difference spectrum was recorded [ΔA(450–490 nm)]. Results (mean values of at least two experiments) are plotted against drug concentrations. C = control.

a Vista 402 data system, a Varian UV-200 detector set at 242 nm and a Berthold LB 506B radioactivity monitor. The testosterone metabolites were separated on a Supelcosil C_{18} column (150 × 4.6 mm, 5 μm; Supelco) preceded by a C_{18} guard column. The column was eluted isocratically (1.3 mL/min) with a mobile phase of methanol/tetrahydrofuran/water (27:5:68) for 12 min, followed by a linear gradient for an additional 23 min to methanol/tetrahydrofuran/water (55.5:7.5:37.0). The radioactivity present in the column effluent was measured directly using Pico-Aqua (Packard) as scintillant at a flow rate of 4 mL/min.

Steroids were identified by comparing the R_f values and retention times with those of standards and/or by gas chromatographic–mass spectrometric analysis as described by Lauwers *et al.* [36].

RESULTS

Spectrophotometric analysis

R 76713 and its enantiomers, R 83839 and R 83842 compete at low concentrations with carbon monoxide for binding to the haem iron of P450 present in human placenta microsomes (Fig. 2). The highest affinity is seen with the (+) enantiomer R 83842.

The higher affinity of R 83842 for the microsomal P450(s) of human placenta microsomes was further proven by studying the stability of the reduced P450–drug complexes. R 76713, R 83839, R 83842 and/or DMSO were added to microsomal suspensions containing P450 isozymes in their oxidized state. Upon addition of the reductant, dithionite, and saturation with CO, a spectrum with an absorption peak at about 450 nm was obtained. In the presence of DMSO the reduced P450–CO complex was stable over the 60-min measurement period (Fig. 3). In the presence of 10^{-8} M of R 83842 or R 76713 ΔA(450–490 nm) decreased by 47 and 37.5%, compared to the control, whereas the (−) enantiomer, R 83839, had no effect on the absorption at 450 nm (Fig. 3). Increasing the drug concentrations up to 10^{-7} M resulted in the formation of stable P450–R 76713 and P450–R 83842 complexes that were not affected by the addition of CO (Fig. 3). Although R 83839 also decreased CO binding (Fig. 3), the complex formed was much less stable as shown by the increase of ΔA(450–490 nm) in function of time. This change represents a partial replacement of R 83839 by CO.

Binding to P450 was further shown by the difference spectra induced by successive additions of R 76713 to a microsomal P450 suspension. In the absence of R 76713 and with androstenedione added to the sample cuvette a characteristic Type I spectrum with an absorption minimum at about 420 nm and absorption maximum at about 390 nm was observed (Fig. 4A). Such an enzyme–substrate spectral change indicates an increase in the high spin state of P450 and suggests the formation of an enzyme–substrate complex [37]. Adding increasing concentrations of R 76713 to microsomal suspensions, previously equilibrated with 1 μM androstenedione induced a difference spectrum with an absorption maximum at 425 nm and a minimum at about 391 nm. The isosbestic point was at about 410 nm (Fig. 4B). Under these conditions, the enzyme in the reference cuvette remained in the high spin state but the enzyme in the sample cuvette was converted to the low spin state [27]. The spectra obtained are almost mirror images of the type I spectral change.

The results shown in Table 1, indicate that R 76713 and its enantiomers have a high affinity for the human placental P450(s) but have almost no effect on other mitochondrial or microsomal P450 isozymes. Indeed, IC_{50}-values were not reached at 10 μM, when the effects of these compounds on P450s in microsomes from bovine adrenal cortex, pig testis, and liver or lung from untreated and/or phenobarbital or 3-methylcholanthrene pretreated rabbits were evaluated. A 50% decrease in ΔA(450–490 nm) was reached at 7.9 μM of R 83839 when added to a mitochondrial suspension of bovine adrenals.

Inhibition of the human placental aromatase

From the interaction with placental microsomal P450 an inhibition of the conversion of androstenedione into oestrogens resulted (Fig. 5). Fifty per cent inhibition was already reached at 2.59 ± 1.37 nM R 76713. The inhibitory effects of R 76713 on the human aromatase were largely determined by its (+) enantiomer, R 83842. Only 1.38 ± 0.64 nM was needed to obtain a 50% inhibition, whereas 44.2 ± 14.2 nM was needed of R 83839.

As already suggested above, R 76713 seems to be able to replace androstenedione from its binding place. As shown in the Lineweaver–Burk-type plots (Fig. 6A–C) of the inhibition of androstenedione aromatization by R 76713 and enantiomers, v^{app} is almost constant whereas $K_m{}^{app}$ varies. This kind of inhibitory effect is normally taken as an indication

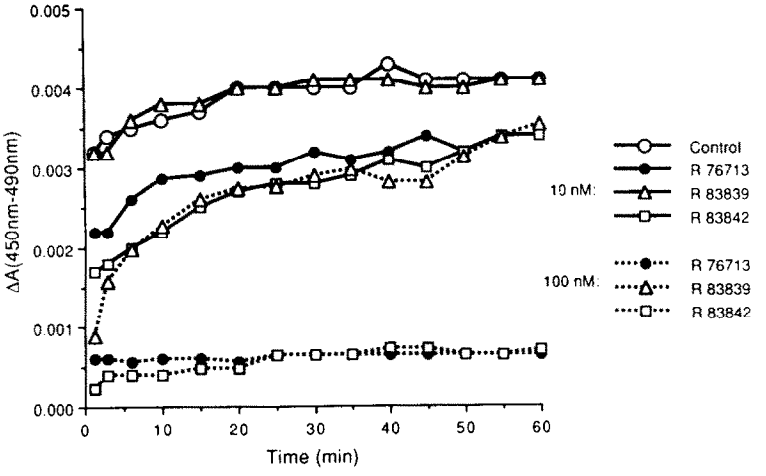

Fig. 3. Data taken from difference spectra obtained with microsomal preparations of human placental microsomes. The microsomal suspensions (P450 content = 0.04 nmol/mL) were divided between the reference and sample cuvettes, a base line of equal light absorbance was established, DMSO (Control) and 10^{-8} or 10^{-7} M of R 76713, R 83839 or R 83842 in DMSO were added to the sample and reference cuvettes. The P450(s) were reduced with dithionite. The sample cuvette was bubbled with CO (30 sec) and tightly closed. The difference spectra were recorded at different time intervals. The differences in absorption (ΔA) at 450 and 490 nm are plotted against time.

of competitive inhibition. The K_i (dissociation constant) value obtained from a replot of the slopes of the Lineweaver–Burk plots of the inhibition by R 76713 (Fig. 6D) was 1.3 nM (mean of four independent experiments). The calculated (Stat View 512+™; BrainPower Inc. U.S.A.) K_i values were 2.4 ± 0.9 nM. The apparent K_m value observed for androstenedione was 35.1 ± 15.6 nM (mean of 10 experiments ± SD). This indicates that R 76713 binds to the placental aromatase at least 13.5 times more strongly than androstenedione. The K_i values obtained from a replot of the slopes of the Lineweaver–Burk plots of the inhibitions by R 83839 (Fig. 6E) and R 83842 (Fig. 6F) were 18 and 0.7 nM. These values again indicate that R 76713's inhibitory effects originate from R 83842. Plotting S/v (S = substrate concentration, v = velocity) against the inhibitor concentration, i, (Cornish-Bowden plot) or $1/v$ against i at different substrate concentrations (Dixon plot) further suggests that there is a competitive part [38] in the inhibition of the human placental aromatase by R 76713 (figures not shown).

Selectivity

As shown in Table 1, R 76713 and its enantiomers have, as compared with P450(s) in human placental microsomes, a low, if any, affinity for P450s in mitochondrial and microsomal membranes. The enantiomer with the lowest effect on the placental P450, R 83839, inhibited CO binding to the P450(s) in bovine adrenal mitochondria. However 50% inhibition only was reached at 7.9 μM (Table 1). R 83839 was also a more potent inhibitor than R 83842 of cortisol synthesis from 11-deoxycortisol in bovine adrenal mitochondria (Table 2). However, 50% inhibition of the 11β-hydroxylase was achieved only at concentrations as high as 43 μM (R 76713), 29 μM (R 83839) and 64 μM (R 83842).

The three compounds also slightly inhibited androgen synthesis from pregnenolone in subcellular fractions from bovine adrenals and rat testis (Table 2). This effect might originate from an effect on the 17,20-lyase (Fig. 7). Using a subcellular fraction (S-10,000) of rat testis and [^{3}H]17α-hydroxy-20-dihydroprogesterone as substrate, 50% inhibition of androgen synthesis (Fig. 7A) was obtained at 1.8 ± 0.3 μM, 2.9 ± 0.5 μM and 1.8 ± 0.5 μM (mean values ± SD) of R 76713, R 83839 and R 83842. This inhibition coincided with an accumulation of the substrate, 17α-hydroxy-20-dihydroprogesterone. The small effect of R 76713 and enantiomers on androgen synthesis in bovine adrenal microsomes resulted in an increased availability of pregnenolone and progesterone for the synthesis of 11-deoxycorticosterone (DOC). At 10 μM the synthesis of DOC increased 1.4–1.7 times as compared with the controls. This suggests that these triazole derivatives do not interfere with the P450-dependent 21-hydroxylase.

Up to 10 μM, these aromatase inhibitors did not inhibit cholesterol synthesis from [^{14}C]mevalonate in rat liver subcellular fractions (S-10,000), cholesterol side-chain cleavage in sonicated bovine adrenal cortex mitochondria, or cholesterol 7α-hydroxylation in female rat liver microsomes (Table 2).

Incubation of rat liver microsomes in the presence of increasing concentrations (0.1 μM up to 10 μM) of the racemic mixture or the two enantiomers did not significantly inhibit the regio- and stereoselective oxydations of [^{14}C]testosterone (Table 3).

DISCUSSION

Using a VGAP alignment programme (alignment with a variable gap penalty) [39] it can be shown that the human aromatase sequence [40], had only 17.9–

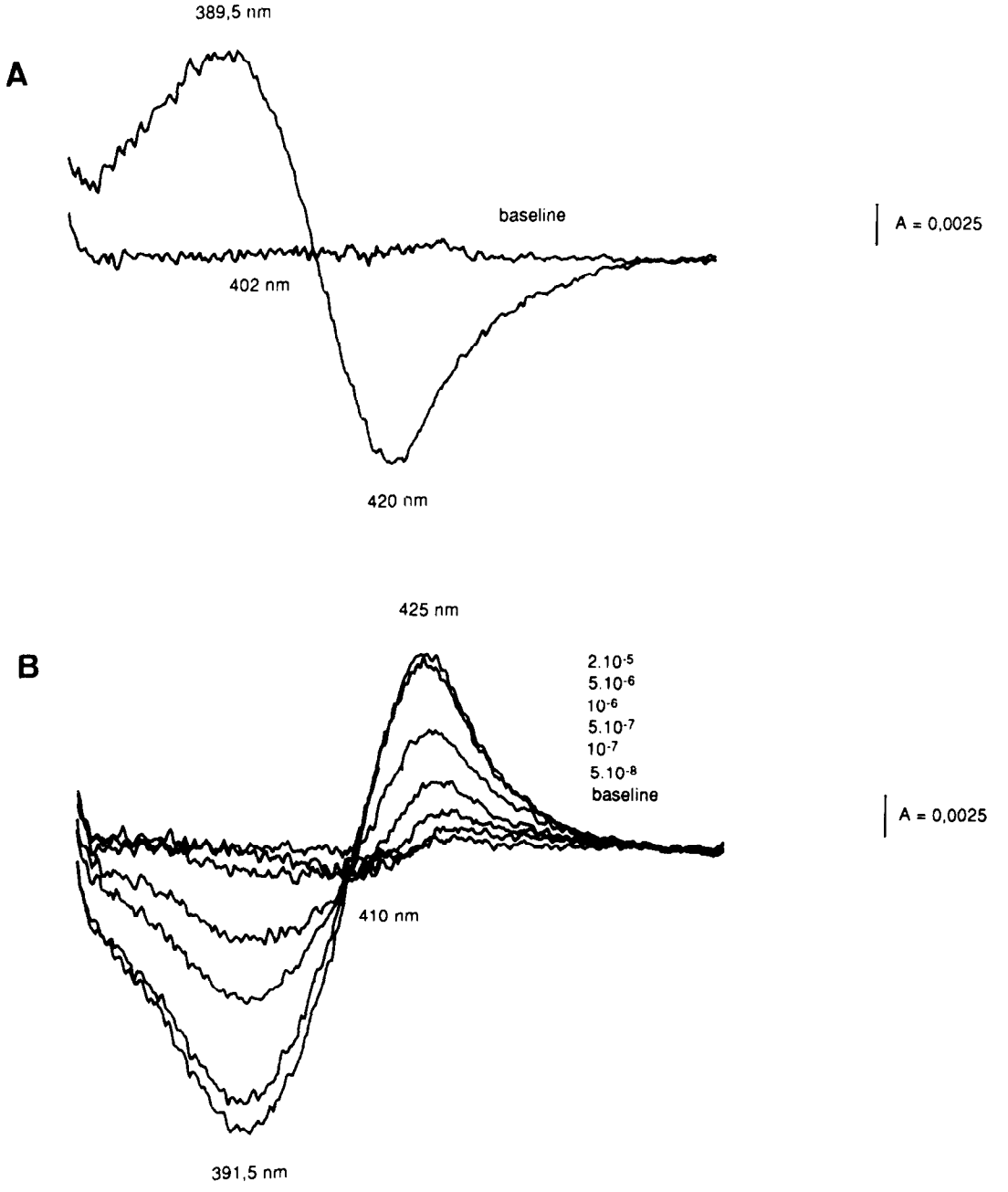

Fig. 4. (A) Androstenedione-induced difference spectrum for human placental microsomes. The sample and reference cuvettes contained 2.1 mg protein/mL (P450 content = 53 pmol/mL). One μM androstenedione was added to the sample cuvette. (B) Difference spectra induced by the addition of increasing concentrations of R 76713 (0.05–20 μM). The sample and reference cuvette contained 2.1 mg protein/mL and 1 μM androstenedione. Isosbestic point = 410 nm.

23.5% identical amino acids in common with the human adrenal mitochondrial 11β-hydroxylase ($P450_{11\beta}$) [41], the human adrenal cholesterol side-chain cleavage ($P450_{SCC}$) [42], the human 17α-hydroxylase ($P450_{17\alpha/17,20\text{-lyase}}$) [43], the mouse $P450_{16\alpha}$ [44] and the rat liver $P4540_a$ [45], $P450_b$ [46], $P450_c$ [47], $P450_g$ [48], $P450_h$ [49] and $P450_p$ [50]. This low sequence identity indicates that $P450_{AROM}$ is a unique form of the P450 superfamily and suggests that it should be possible to synthesize a rather specific aromatase inhibitor.

The results presented in this paper prove that the triazole derivative, R 76713 is a potent inhibitor of the human placental aromatase. Fifty per cent inhibition is already achieved at 2.59 $\pm$ 1.37 nM. This value corresponds very well with the 5.1 and 3 nM of R 76713 needed for the rat ovarian aromatase [51] and in primary cultures with follicle-stimulating

Table 1. Effects of R 76713 and enantiomers on binding of CO to P450*

Species	Organ	Membranes	IC_{50} values ($\times 10^{-8}$ M) R 76713	R 83839	R 83842
Human	Placenta	Microsomes	2.95	4.00	1.79
Bovine	Adrenal	Mitochondria	>1000	788	>1000
		Microsomes	>1000	>1000	>1000
Piglet	Testis	Mitochondria	>1000	>1000	>1000
		Microsomes	>1000	>1000	>1000
Rabbit	Liver (basal)	Microsomes	>1000	>1000	>1000
	Liver (PB)	Microsomes	>1000	>1000	>1000
	Liver (3MC)	Microsomes	>1000	>1000	>1000
	Lung	Microsomes	>1000	>1000	>1000

* The membrane fractions were diluted in 0.1 mM potassium phosphate buffer (pH 7.4) to obtain a P450 content of 0.04 nmol/mL (human placenta) or 0.1 nmol/mL (all other fractions). The suspension was divided between the reference and sample cuvettes, drugs and/or solvent were added to both cuvettes and the P450s were reduced with a few grains of sodium dithionite. The sample cuvette was bubbled for 30 sec with CO and then tightly closed. The difference spectrum was recorded 45 sec later. ΔA(450–490 nm) were determined. By weight non-linear regression procedure, a sigmoidal dose–response model was fitted to the individual observations and the corresponding IC_{50} values calculated. IC_{50} value: drug concentration needed to obtain 50% decrease in ΔA(450–490 nm). PB: phenobarbital; 3MC; 3-methylcholanthrene.

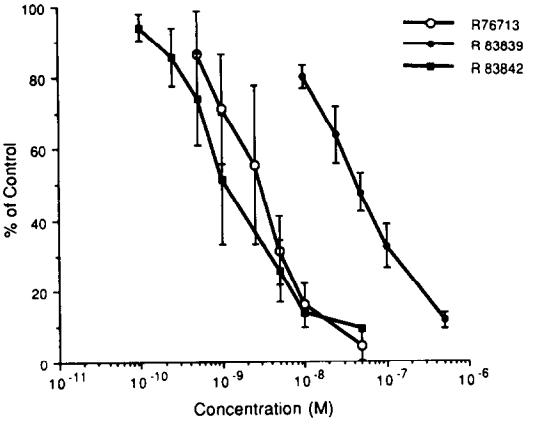

Fig. 5. Effects of R 76713 and enantiomers on the synthesis of oestrogens (oestrone plus oestradiol) from [4-^{14}C]androstenedione by human placental microsomes. Under control conditions 143.9 ± 11.0 pmol/min/mg protein oestrone and 13.0 ± 3.9 pmol/min/mg protein oestradiol (N = 16) are formed. Results (mean values ± SD of at least four experiments) obtained after 6 min of incubation in the presence of R 76713, R 83839 or R 83842, expressed as per cent of control, are plotted against concentration. IC_{50} values are 2.59 ± 1.37 nM, 44.2 ± 14.2 nM and 1.38 nM, respectively.

hormone (FSH) stimulated rat granulosa cells [52] and with the 4.5 nM of CGS 16949A [4-(5,6,7,8-tetra-hydrimidazo[1,5-a]pyridin-5-yl)benzonitrile monohydrochloride) needed to get 50% inhibition of the placental aromatase activity [29]. The potency of R 76713 has also been proven *in vivo*: single oral doses of 0.05 mg/kg lower plasma oestradiol levels of pregnant mare serum gonadotropin (PMSG)-primed rats by more than 90% [52].

This inhibitory effect might originate from R 76713's high affinity for the placental P450. Indeed, when added at a 0.1 μM concentration to a microsomal suspension of human placental microsomes, the R 76713–P450 complex formed cannot be replaced by a CO–P450 complex suggesting that, as has been found with azole antifungals such as itraconazole [25, 53], R 76713 not only coordinates by its triazole nitrogen (N-4) to the haem iron but that its N-1-substituent also occupies a lipophilic region(s) of the apoprotein moiety of P450. The reversed type I spectral change observed when increasing concentrations of R 76713 are added to microsomal suspensions first equilibrated with androstenedione suggests a displacement of the substrate from the binding site on P450 [37]. Furthermore, the Lineweaver–Burk, Dixon and Cornish-Bowden plots indicate that there is a competitive part in the inhibition of the human placental aromatase by R 76713. Therefore, the portion of the apoprotein occupied by R 76713 might be part of the substrate binding place. However, it is also possible that an interaction of the N-1-substituent with the apoprotein changes its conformation in such a way that substrate binding is infringed. Whatever the interaction may be, these results indicate that the interaction of this non-steroidal inhibitor with the P450-dependent reaction is complex: firstly the inhibitor eliminates the catalytic potential of the enzyme by inhibiting oxygen binding and activation and secondly it affects substrate binding.

Double reciprocal plots have also shown the competitive nature of the inhibition by R 76713 in rat ovarian homogenates [51] and by CGS 16949 [29], aminoglutethimide [26], 4-cyclohexylaniline [27, 29] and 10-oxirane and 10-thiirane substituted androgens [54] of human placental aromatase activity. The difference spectra induced by the addition of R 76713 to the high spin, substrate bound form of the microsomal P450 equilibrated with androstenedione resemble those obtained with *d*-aminoglutethimide, 4-cyclohexylaniline [27], 10-oxiranyl- and 10-thiiranyl-4-androstene-3,17-dione [54] when added to

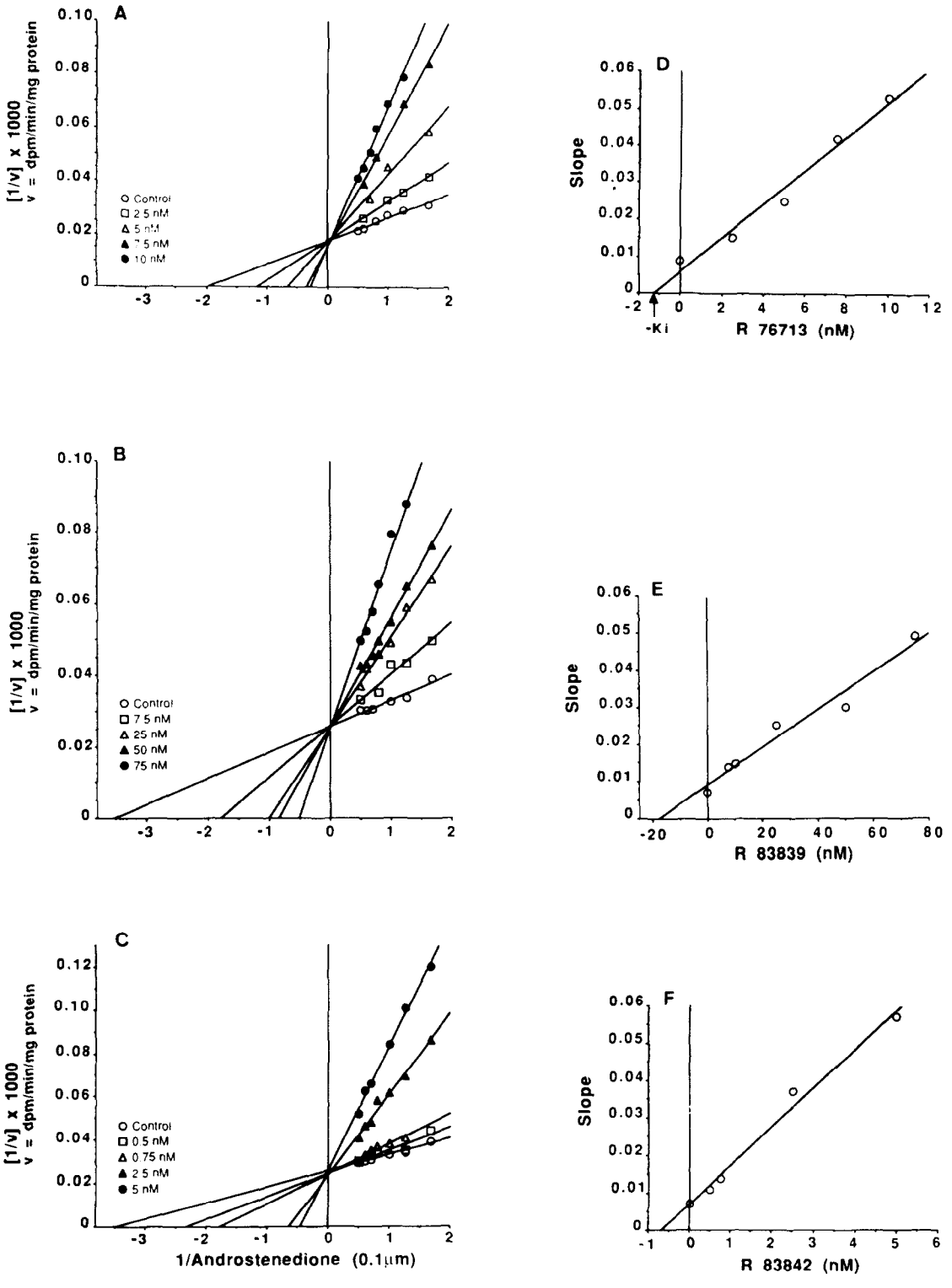

Fig. 6. (A) Kinetic analysis of the mechanism of inhibition of androstenedione aromatization in human placental microsomes by R 76713 (Lineweaver–Burk-type plot). The results presented are mean values of four different experiments. The protein content was 25 μg/mL; incubation time: 6 min; v is given as dpm/min/mg protein. (B and C) Kinetic analysis of the mechanism of inhibition of androstenedione aromatization in human placental microsomes by R 83839 (B) and R 83842 (C). The results presented are mean values of two different experiments. (D–F) Slope of lines in double reciprocal plot vs inhibitor concentration. The K_i values obtained with R 76713, R 83839 and R 83842 are: 1.3 nM, 18 nM and 0.7 nM, respectively.

Table 2. Effects of R 76713 and enantiomers on P450-dependent reactions

Organ*	Substrate tested†	Product formed	% Inhibition at 10^{-5} M‡ R 76713	R 83839	R 83842
Rat liver (S-10,000)	Mevalonate	Cholesterol	0.0 (3)	0.0 (2)	0.0 (2)
Rat liver (micros.)	Cholesterol	7α-OH-cholesterol	0.0 (2)	0.0 (1)	0.0 (1)
Bovine adrenals (mitoch.)	Cholesterol	Preg.	0.0 (3)	0.0 (2)	0.0 (2)
Rat testis (S-10,000)	Preg.	Testo.	33.4 ± 15.2 (5)	20.6 ± 3.0 (4)	23.0 ± 2.9 (4)
Bovine adrenals (mitoch.)	11-Deoxy-cortisol	Cortisol	17.1 ± 6.1 (4)	14.3 ± 3.4 (4)	4.5 ± 4.1 (4)
Bovine adrenals (micros.)	Preg.	DHEA + A.dione	25.4 (3)	24.0 (2)	19.6 (2)

* S-10,000 supernatant of a 10,000 *g* centrifugation; micros., microsomes; mitoch., mitochondria.
† DHEA, dehydroepiandrosterone; A.dione, androstenedione; Testo., testosterone; Preg., pregnenolone.
‡ Results are mean values ± SD; figures in parenthesis = number of experiments.

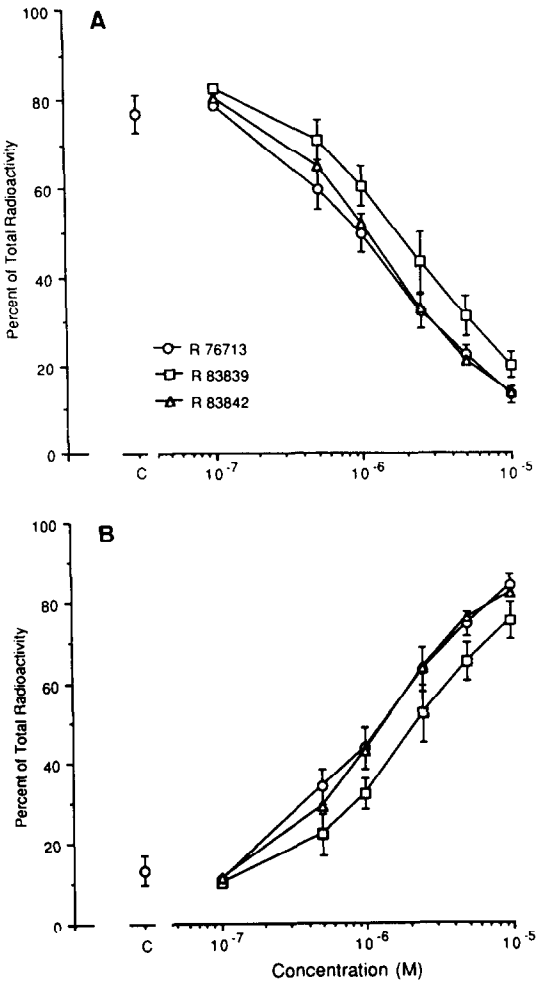

Fig. 7. Effects of R 76713 and enantiomers on the synthesis of androgens (androstenedione, DHEA, testosterone) from [^{3}H]17α-hydroxy-20-dihydroprogesterone by a S-10,000 fraction of Wistar rat testis. S-10,000 fractions were incubated for 2 hr at 37° in the presence of substrate, a NADPH generating system [26] and increasing concentrations of the triazole derivatives and/or DMSO (C). (A) Inhibition of androgen synthesis. (B) Accumulation of 17α-hydroxy-20-dihydroprogesterone.

cholate-extracted human placental microsomes [27] or purified aromatase [54]. The list contains non-steroidal as well as steroidal inhibitors, all of them binding through their amine, nitrogen, oxygen or sulfur atom to the haem iron thereby inhibiting oxygen binding, activation and thus the P450 catalysed hydroxylations. Therefore, it is possible that the competitive nature of inhibition originates from the binding to the haem iron and that binding to the apoprotein moiety stabilizes the interaction.

Most of R 76713's inhibitory effects on the aromatase activity originates from R 83842: compared to the (−) enantiomer R 83839, R 83842 has a greater effect on CO-binding to the haem iron and forms a much more stable complex with the placental P450. About 32-times (Table 4) less compound is needed to achieve 50% inhibition of the aromatization of

Table 3. Effects of R 76713 and enantiomers (10 μM) on testosterone metabolism in male Sprague–Dawley rat liver microsomes

Testosterone metabolite formed†	% of Total radioactivity*			
	Control	R 76713	R 83839	R 83842
2α—OH—	5.0 ± 1.3 (12)	3.6 ± 2.1 (4)	4.2 ± 1.4 (4)	4.1 ± 0.5 (4)
6α—OH—	3.2 ± 1.2	2.0 ± 0.6	3.1 ± 2.4	2.0 ± 0.4
6β—OH—	16.4 ± 8.5	10.5 ± 5.8	8.7 ± 3.3	11.8 ± 9.8
7α—OH—	9.6 ± 2.8	10.0 ± 8.4	8.6 ± 1.2	8.0 ± 2.1
16α—OH—	15.8 ± 5.0	13.0 ± 6.4	13.8 ± 5.3	16.3 ± 4.8
16β—OH—	9.5 ± 1.6	8.2 ± 1.4	7.4 ± 2.9	7.6 ± 2.2
17—O—	2.2 ± 1.3	4.0 ± 0.6	2.4 ± 1.3	3.6 ± 0.6

* The incorporation of radioactivity from [4-^{14}C]testosterone into hydroxylated and oxidated testosterone metabolites by liver microsomes from male rats pretreated with phenobarbital is measured in the presence of increasing concentrations (0.1–10 μM) of R 76713, R 83839 or R 83842 (the results obtained in the presence of 10 μM are given only). Controls are incubated in the presence of the solvent, DMSO. Results are given as per cent (mean ± SD) of the total radioactivity recovered in the extracts. Figures in parenthesis are numbers of experiments.

† Steroids are separated by TLC and HPLC and identified by comparing the R_f values and retention times with those of standards and/or by gas chromatographic–mass spectrometric analysis.

Table 4. Effects of R 76713, enantiomers, and reference compounds on human placental aromatase (microsomes)

Compound	Relative potency†
R 83842	1029
R 76713	548
R 83839	32
4-Hydroxyandrostene-3,17-dione*	34
Aminoglutethimide	1

* Obtained from Dr A. M. H. Brodie (Baltimore, U.S.A.).

† The IC_{50} value (1.42 μM) obtained with aminoglutethimide is used as standard.

androstenedione, and the K_i value of R 83842 is about 26-times lower than that of R 83839. These results are indicative of a strong stereospecificity for binding.

Under the present experimental conditions, R 83842 is 30- and 1029-times more active than 4-hydroxyandrostene-3,17-dione and aminoglutethimide, respectively (Table 4).

The stereo- and regioselective metabolic attack of testosterone by P450 isozymes is well known (for a review see Ref. 55). For example, in rat liver, $P450_a$ (IIA1) catalyses the oxidation of testosterone to one major product, 7α-hydroxytestosterone and one minor product, 6α-hydroxytestosterone, $P450_b$ (IIB1) catalyses the hydroxylations at 16α and 16β; the 6β-hydroxylation is catalysed by $P450_c$ (IA1) and $P450_g$ (IIC13) and the 2α- and 16α-hydroxylations by $P450_h$ (IIC11) [55]. $P450_h$ and $P450_b$ also oxidize testosterone at the 17β-position to form androstenedione. Up to 10 μM no statistically significant effect on the P450-dependent oxidations of testosterone is observed with R 76713 and its enantiomers. These results suggest that even at this high dose, these triazole derivatives do not interact with major liver P450 isozymes.

Up to 10 μM, R 76713 and its enantiomers are devoid of any effect on the P450-dependent cholesterol synthesis, cholesterol side-chain cleavage and the 7α-hydroxylation of cholesterol, the first step in bile acid synthesis. They do not interfere with the 21-hydroxylation of progesterone. At 10 μM, R 76713 has a slight effect on the adrenal 11β-hydroxylase, which originates from R 83839, i.e. the enantiomer with the lowest effect on aromatase activity. This small effect indicates that, as could be expected from the spectrophotometric studies, that these compounds have a low affinity for $P450_{11\beta}$, a P450 that not only catalyses the hydroxylation of 11-deoxycortisol and 11-deoxycorticosterone but also has 18-hydroxylase and aldehyde synthase activities and thus mediates the synthesis of aldosterone [56]. Studies on human adrenal cells [57] and *in vivo* on rats [58] confirm the low effect of R 76713 on aldosterone synthesis and suggest that R 76713 differs from another new aromatase inhibitor CGS-16949 A, which has shown to be a potent inhibitor of the 11β-hydroxylase and aldosterone production by human adrenal cells *in vitro* [59].

R 83842 (≥0.5 μM) is, as compared to R 83839, a more potent inhibitor of the 17,20-lyase in subcellular fractions of rat testis and of androgen synthesis from pregnenolone by bovine adrenal microsomes. Fifty per cent inhibition of the testes, 17,20-lyase is achieved at 1300-times the concentration needed to reach this inhibition level for the human placental aromatase. Although R 83842 and R 76713 are less powerful inhibitors of this key-step in androgen synthesis than ketoconazole [60], this property might be of some help to reduce the amount of accumulating androgens.

The preceding results and discussion demonstrate that R 76713 and its (+) enantiomer, R 83842, are potent and selective inhibitors of the P450-dependent aromatase and suggest that these compounds might be of benefit in the treatment of oestrogen-dependent diseases. This study also indicates that P450 systems can be exploited in the search for new possibilities in medical treatment.

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