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Inhibitors of Human and Rat Testes Microsomal 17β-Hydroxysteroid Dehydrogenase (17β-HSD) as Potential Agents for Prostatic Cancer

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In a screening programme for inhibitors of human testis 17β-hydroxysteroid dehydrogenase (17β-HSD type 3), as potential agents for the treatment of hormone-dependent prostatic cancer, we have used crude human testis microsomal 17β-hydroxysteroid dehydrogenase as a convenient source of the enzyme. Crude human enzyme was shown to have a similar substrate profile to recombinant Type 3 17β-HSD from the same source as determined by the low K_m/V_{max} ratio for the reduction of androstenedione compared to the oxidation of testosterone, and a low level of activity in reduction of oestrone. Screening of a wide range of compounds of different structural types as potential inhibitors of the microsomal enzyme in the reduction step revealed that certain p-benzoquinones and flavones/isoflavones were potent inhibitors of the enzyme, diphenyl-p-benzoquinone (2.7 μM), phenyl-p-benzoquinone (5.7 μM), 7-hydroxyflavone (9.0 μM), baicalein (9.3 μM) and biochanin A (10.8 μM). Some structure–activity relationships within the flavone/isoflavone series are discussed. Studies with rat testis microsomal 17β-HSD showed that it differed from the human enzyme mainly in its greater ability to accept oestrone as substrate and the pH-optimum for oxidation of testosterone. It was found to be much less sensitive to inhibition by the compounds studied so negating it use as a more readily available tissue for the screening of potential inhibitors.

Keywords: 17β-Hydroxysteroid dehydrogenase, 17β-HSD, Human testis microsomes, Rat testis microsomes, Flavones/isoflavones, Benzoquinones, Prostatic cancer

INTRODUCTION

Androgen ablation strategies for the treatment of prostatic cancer in the early hormone-dependent stage are castration, androgen receptor antagonism and blockade of androgen synthesis (cytochrome $P450_{17\alpha}$ and 5α-steroid reductase inhibitors as well as GnRH agonists). Prostatic cancer growth is dependent on dihydrotestosterone produced by the action of 5α-steroid reductase on testosterone which is derived from the weak androgen androstenedione by the action of 17β-hydroxysteroid dehydrogenase (17β-HSD) Scheme 1. Thus 17β-HSD is central to the conversion of weak androgen to its potent form and inhibitors of testicular 17β-HSD could provide novel agents for the treatment of prostatic cancer.

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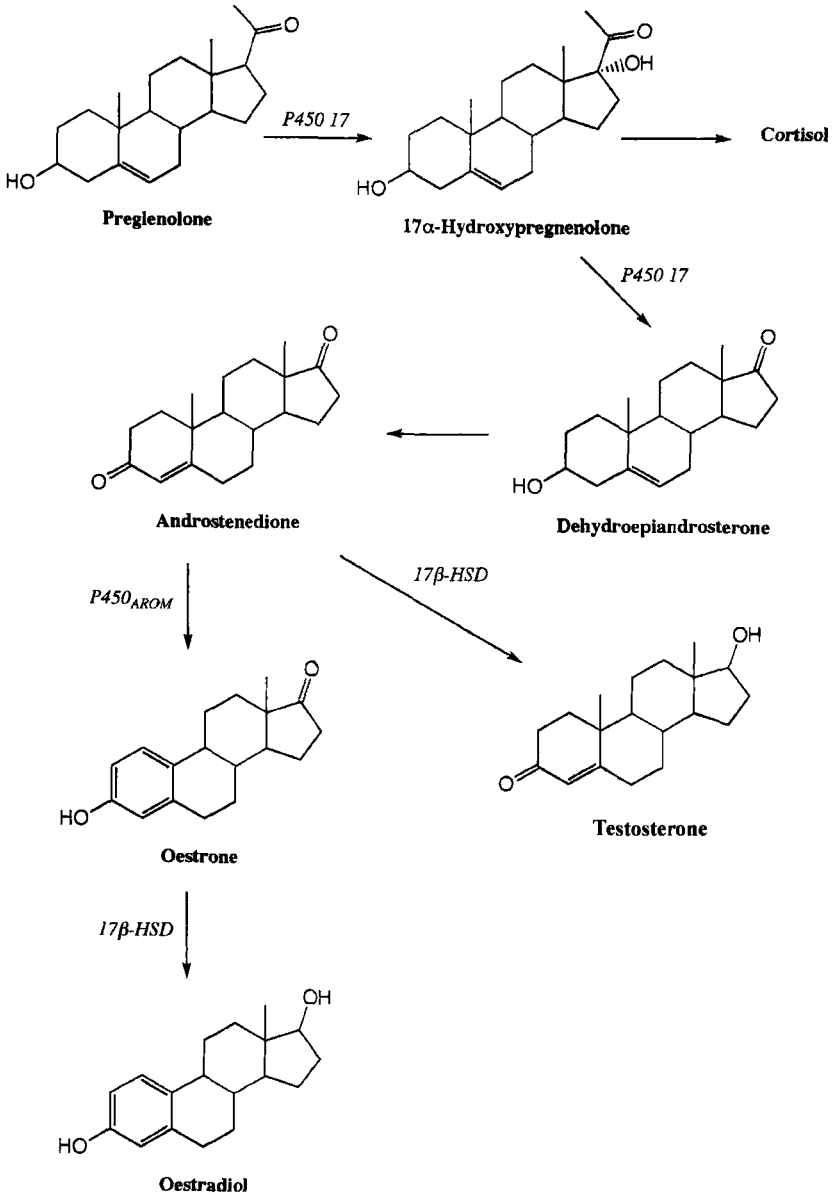
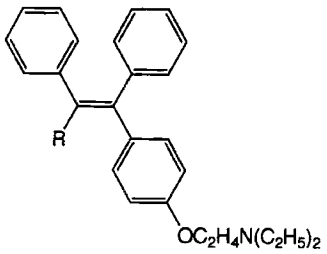
17β-HSD exists in multi isoenzyme forms. Types 1–7 have been identified in various tissues species using molecular biological tools.

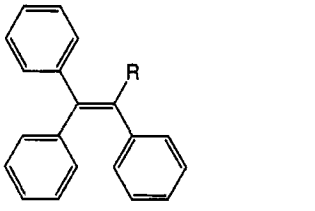
17β-HSD type 1 human placenta soluble (cytosolic) enzyme[1–4] shows a high degree of specificity for C18 steroids (oestrone, E_1; oestradiol, E_2) as C19 (androstenedione, testosterone) and C21 (dihydroprogesterone) steroids are bound as substrates or inhibitors with much lower affinity than oestradiol.[5,6] This is confirmed by the fact that the cytosolic enzyme presents a high E_2/T activity ratio[7] confirming the tendency of the cytosolic form to be more specific for oestrogens as suggested by Thomas *et al.*[8] The type 1 soluble enzyme from placenta was about equally reactive with E_1 and E_2 when activity was assessed on



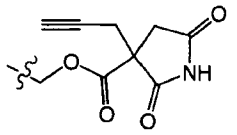
SCHEME 1 Steroidogenesis pathway.



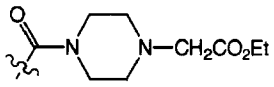
(1) R = Et ; Tamoxifen
(2) R = Cl ; Clomiphene

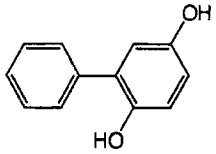


(3) R =

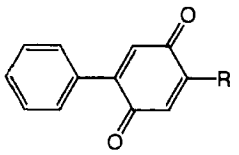


(4) R =

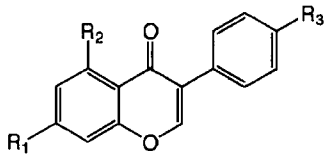


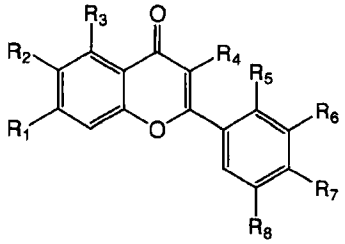


(7) 2-Phenylhydroquinone



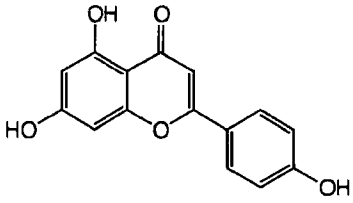
(5) R = H ; Phenyl-*p*-benzoquinone
(6) R = C_2H_5 ; Diphenyl-*p*-benzoquinone





(all substituents are H unless otherwise stated)

(8) $R_1 = R_2 = R_3 = OH$; Genistein
(9) $R_1 = R_2 = OH$, $R_3 = OCH_3$; Biochanin A



(18) Naringenin

(10) $R_2 = OH$; 6-Hydroxyflavone
(11) $R_1 = OH$; 7-Hydroxyflavone
(12) $R_1 = R_3 = OH$; Chrysin
(13) $R_1 = R_3 = R_7 = OH$; Apigenin
(14) $R_1 = R_2 = R_3 = OH$; Baicalein
(15) $R_1 = R_4 = R_7 = R_8 = OH$; Fisetin
(16) $R_1 = R_3 = R_4 = R_5 = R_7 = OH$; Morin
(17) $R_1 = R_3 = R_4 = R_6 = R_7 = R_8 = OH$; Myricetin

subcellular fractions,[7] while the recombinant type 1 enzyme transiently expressed in cultured mammalian cells almost preferentially catalysed the reduction of E_1.[9,10]

The type 2 isozyme from placental microsomes[11] recognises C18, C19 and C21 steroids as substrates with comparable affinities. It possesses 20α-HSD activity. It is characterised

by a low E_2/T ratio[7] and preferentially catalyses the oxidative step.[11,12,13]

cDNA corresponding to a 17β-HSD type 3 has been isolated.[14] The 17β-HSD type 3 from human testes microsomes shows only 23% sequence identity with other 17β-HSDs.[15,16] It uses NADPH as cofactor, and favours testosterone formation from androstenedione[14] with a low level of reduction of E_1 into E_2.[12] Other 17β-HSDs may be present in this tissue. Blomquist (1995) reports an E_2/T ratio for testicular activity in the cytosol very much smaller than the ratio (>100) characteristic of type 1 and an E_2/T ratio in microsomes characteristic of type 2 which suggest the possibility of the presence of the latter. However, Casey *et al.*[18] have found no evidence of type 2 mRNA species in testes.

Other 17β-HSD isozymes are known; a type 4 has been identified in cytoplasmic vesicles of porcine endometrial cells[19,20] and in peroxisomes[21] and its human counterpart has been identified, type 5 17β-HSD isozyme has been reported in human placenta that possesses 20α-HSD activity,[12] the isolation of a cDNA encoding a type 6 isozyme, present in the rat ventral prostate and in liver has been reported[22] and recently, a type 7 has been isolated from the HC11 cell line from mouse mammary gland.[23]

This work is concerned with the discovery of inhibitors of human testis type 3 isozyme as potential agents for the treatment of hormone-dependent prostatic cancer using a crude microsomal testis enzyme for screening. This was characterised as Type 3 by kinetic parameters, pH-rate profiles towards androstenedione and testosterone, its activity towards the oestrogens, oestrone and oestradiol and the equilibrium for the interconversion androstenedione testosterone. Inhibition of androstenedione reduction was examined for a range of compounds from many structural types. Rat testes microsomal enzyme was similarly studied and compared with the human form since a suitable comparison would lead to the use of the rat enzyme as a more readily available tissue for the screening of potential inhibitors.

MATERIALS AND METHODS

[1,2,6,7-^{3}H] 4-Androstene-3β, 17β-dione (86.4 Ci/mmol–37 MBq/mL), [1,2,6,7-^{3}H] testosterone (85 Ci/mmol, 37 MBq/mL), [4-^{14}C] oestrone (52.5 mCi/mmol–0.74 MBq/mL), [4-^{14}C] oestradiol (55.5 mCi/mmol–0.74 MBq/mL), [4-^{14}C] testosterone (57.3 mCi/mmol–1.5 MBQ/mL) and [4-^{14}C] 4-androstene-3β, 17β-dione (53.9 mCi/mmol–0.74 MBq/mL) were purchased from NEN-Dupont UK (Stevenage, Herts). [2,4,6,7-^{3}H] Oestrone (92 Ci/mmol–37 Mbq/mL) and [2,4,6,7-^{3}H] oestradiol (94 Ci/mmol–37 MBq/mL) were from Amersham plc (Amersham, Bucks).

Oestrone, 4-androstene-3, 17-dione, testosterone and oestradiol were obtained from Sigma Chemical Co. (Poole, UK). D-Glucose-6-phosphate dehydrogenase (suspension in ammonium phosphate) was obtained from Boehringer Mannheim (Mannheim, FDR). Scintillation fluid was optiphase Hisafe from Fisons Chemicals (Loughborough, UK). Laboratory reagents were of Analar grade (Fisons Chemicals). The scintillation counter used was an LKB Wallac, 1217, Rackbeta. An MSC Ultracentrifuge with 8 × 50 ml fixed angle rotor, Ultracentrifuge Sorvall OTD Combi 1 with 8 × 30 ml fixed angle rotor and Potter-Elvejhem homogeniser were used. TLC plates F_{254} from Sigma Chemical Co (Poole, U.K.) were used.

The sources of the purchased flavones/isoflavones were: daidzen (ICN Biomedicals, Thames, Oxfordshire), 3-hydroxyflavone (Lancaster, Morecombe, Lancs), 6- and 7-hydroxyflavone (Aldrich Chemical Co., Gillingham, Dorset), fisetin, myricetin (Fisher Scientific U.K., Loughborough, Leicestershire). The remaining compounds were from Sigma Chemical Co. (Poole, Dorset) or synthesised in our laboratories.

HUMAN TESTICULAR MICROSOMES

Preparations

Testes were obtained from a 65 years old patient undergoing orchidectomy with prostatic cancer. The testes were cooled, mixed with phosphate buffer (50 mM, pH 7.45) containing sucrose (0.25M), (3 mL per g of tissue), crushed in a mixer and then homogenised using an Ultra-Turrax homogeniser. The homogenised tissue was then processed by the method of Al-Hamrouni *et al.*[24] except that the microsomal pellet was suspended in phosphate buffer (50 mM, pH 7.45) and homogenised before distribution and storage at −80 °C.

Assay for Reduction of Androstenedione

A solution of [1,2,6,7-^{3}H]-andro stenedione and androstenedione (0.5 μM final concentration) was incubated at 37 °C for 30 min with the human testes microsomal preparation, phosphate buffer, (50 mM, H 7.4) and NADPH generating system (50 μL) (NADP 0.0086 g, G6P 0.028 g, G6PD 10 IU in 1 mL phosphate buffer). Diethyl ether was then added to the tubes and [^{14}C]-testosterone in ethanol (50,000 dmin^{-1}) was introduced into each tube as internal standard. The steroids were extracted with diethyl ether (3 × 2 mL), the combined ether phase evaporated and 30 L of acetone added. The acetone solutions containing substrate and product were spotted on TLC plates. Unlabelled testosterone and androstenedione standards (5 mg/mL) were then run on the same plate. After separation using the solvent system:chloroform/ethyl acetate/methanol (85/12.5/2.5), testosterone and androstenedione spots were located under UV (254 nm). The product spots were then cut out, 2 mL of scintillation fluid and 1 mL of acetone were added and ^{3}H and ^{14}C radioactivity counted in the scintillation counter. Comparison between the ^{3}H radioactivity initially introduced in each tube as substrate ^{3}H and the radioactivity detected as ^{3}H product, with the aid of the ^{14}C product as internal standard, allowed an accurate assessment of the rate of conversion of substrate to product by taking into account the efficiency of the product recovery.

pH-Dependence

Labelled androstenedione (0.25 μM final concentration) was incubated, in duplicate, with 0.106 mg/mL of human testes microsomes and NADPH cofactor (500 μM theoretical concentration before introduction into the medium) at 37 °C for 30 min in several media; pH 4.9–5.5 (0.1 M potassium hydrogen phthalate), pH 6.0–8.0 (0.1 M potassium dihydrogen phosphate) and 8.5–11.0 (0.1 M borax), and the percentage conversion measured.

Figure 1 shows that the optimum activity was centred at pH 6.5.

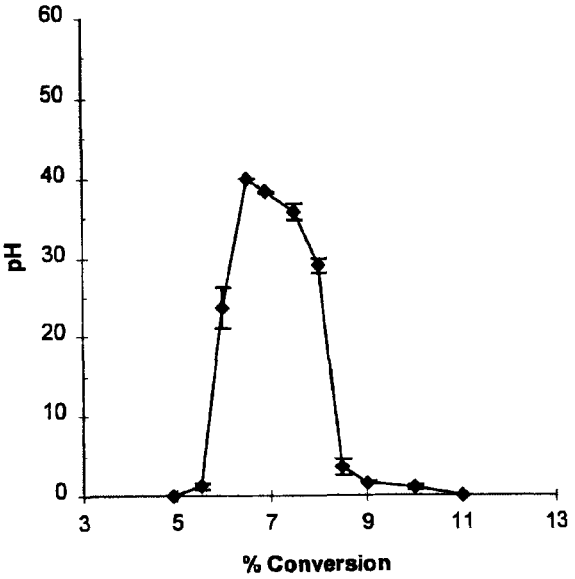


FIGURE 1 pH-Dependence of the reduction of androstenedione (0.25 μM) to testosterone by 17β-HSD from human testes microsomes preparation. Data are presented as the mean of duplicate samples and error bars show the spread of individual values.

K_m *Value*

The assay was performed in duplicate with 0.106 mg mL^{-1} protein and 0.25–7.5 μM labelled and unlabelled substrate for 30 min. The results plotted by Lineweaver-Burk, Hanes-Woolf and Eadie-Hofstee plots (not shown) gave overall K_m and V_{max} values of 0.28 ± 0.06 μM and 0.072 ± 0.014 nmol · min^{-1} mg^{-1}.

Inhibition Studies

The general method described was followed where a solution of [1,2,6,7-^{3}H] andostenedione and androstenedione (0.5 μM and 2.0 μM final) in propylene glycol was incubated, in duplicate, with the microsomal preparation (0.106 mg/mL), in phosphate buffer for 30 min at 37 °C. All compounds (200 μM final) were added in organic solvent (10 μL). Controls in the absence of inhibitors containing organic solvent (10 μL of ethanol or DMSO) were also run. Percentage inhibition was given by 100 – [(% conversion inhibitor/% conversion of control) × 100]. The results summarised in Table I represent the mean percentage of inhibition of duplicate samples where the spread was < 5%. IC_{50} values were determined using a range of inhibitor concentrations in ethanol (10 μL) and a single substrate concentration (2 μM) and determined graphically from a plot of $\log_{10}$[Inhibitor concentration] vs % inhibition using Cricket Graph™ 1.3.

TABLE I Inhibitors of human testes microsomal 17β-HSD for the reduction of androstenedione at pH 7.45

	Compounds	*% Inhibition**	*IC50* (μM)**
1	Tamoxifen	100	98.1
2	Clomiphene	94.4	76.2
3		86.7	9.15
4		52.1	Nd
5	Phenyl-p-benzoquinone	100	5.7
6	Diphenyl-p-benzoquinone	99.0	2.7
7	2-Phenylhydroquinone	100	14.8
8	Genistein	77.8	30.3
9	Biochanin A	89.1	10.8
10	6-Hydroxyflavone	91.3	16.4
11	7-Hydroxyflavone	94.1	9.0
12	Chrysin	79.1	Nd
13	Apigenin	78.2	21.3
14	Baicalein	96.0	9.3
15	Fisetin	93.8	32.4
16	Morin	52.0	Nd
17	Myricetin	97.0	100.5
18	Naringenin	100	16.4

* Compounds at 200 μM, androstenedione at 0.5 μM; ** androstenedione at 2.0 μM. nd = not determined. The values are the means of duplicate samples.

Assay for Oxidation of Testosterone

The general method for reduction of androstenedione was followed except that [1,2,6,7-^{3}H] testosterone (2.0 μM) and 50 μL of a solution of NADP (0.0086 g) in phosphate buffer (1 mL) was used. ^{14}C Androstenedione solution in ethanol (10 μL corresponding to 50,000 $dmin^{-1}$) was used as internal standard.

pH-Dependence

Labelled testosterone (2.0 μM) was incubated with 0.16 mg/mL of rat testes microsomes at 37 °C for 30 min at pH 4.9, pH 6.2 and 7.0, pH 7.45 (0.1 M phosphate buffer) and pH 8.6–10.9 (0.1 M borax).

Figure 2 shows that the optimum pH for oxidation was centred at pH 7.0.

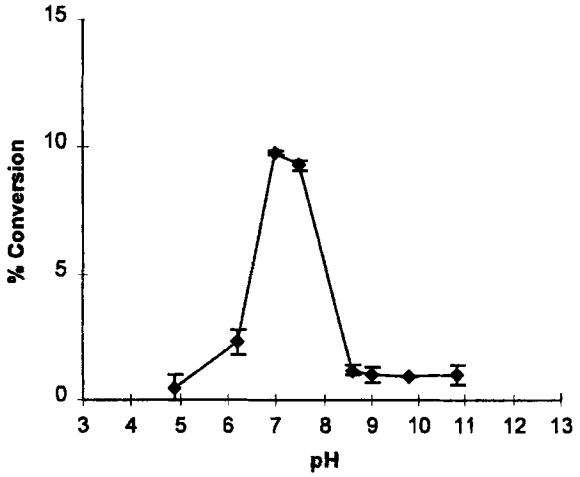


FIGURE 2 pH-Dependency of oxidation of testosterone (2 μM) by 17β-HSD from human testes microsomes. Data are presented as the mean of duplicate samples and error bars show the spread of individual values.

K_m Value

The K_m determination was performed by incubating, in duplicate, human testes microsomes (0.160 mg/mL) with a range of testosterone concentrations (0.5–50 μM) under experimental conditions described previously.

The K_m determined by Lineweaver-Burk, Hanes-Woolf and Eadie-Hofstee plots was 17.2 ± 2.4 μM, and the V_{max} was 0.322 ± 0.053 nmol·min^{-1} mg^{-1}.

Equilibrium for the Interconversion Androstenedione ⇔ Testosterone

Solutions of [^{3}H]-androstenedione and cold androstenedione (2.0 μM), and [^{14}C] testosterone and cold testosterone (2.0 μM) in propylene glycol were incubated with the microsomal preparation (0.160 mg/mL final concentration) NADPH (500 μM) and NADP (500 μM) in phosphate buffer (1 mL final volume) at 37 °C for 30 min. The separation and analytical methods were the same as previously described except that an internal standard was not used.

The results showed that the reduction reaction ($21.3 \pm 0.3\%$, $n = 3$) was favoured over the oxidation reaction ($14.7 \pm 0.5\%$) under these conditions.

Assay for the Reduction of Oestrone

A mixture of [2,4,6,7-^{3}H] oestrone and oestrone (1.0 μM final concentration) was incubated, in duplicate, with various concentrations of human testes microsomes preparation (0.053–0.213 mg/mL) NADPH generating system (50 μL), and phosphate buffer to give 1.0 mL at 37 °C for 30 min and processed as described previously except that labelled [^{14}C]-oestradiol (50000 dmin^{-1}) was used as internal standard, and oestradiol and oestrone (5 mg/mL) were used as marker steroids for TLC.

K_m Value

Labelled and unlabelled oestrone over two concentration ranges (0.5–5.0 μM and 0.5–15 μM) were incubated, in duplicate, with human testes microsomes (0.213 mg/mL) and NADPH generating system (50 μL) at pH 7.45 and 37 °C for 30 min. As no flattening of the curve in the expected hyperbolic manner was obtained up to a substrate concentration of 15 μM on the Michaelis-Menten graph, the work with this system was discontinued. The K_m and V_{max} estimated were 11.6 ± 1.2 μM and 0.231 ± 0.019 nmol·min^{-1}mg^{-1} respectively.

Assay for the Oxidation of Oestradiol

[2,4,6,7–^{3}H]-oestradiol (1.0 μM) was incubated at pH 7.45 for 30 min with human testicular microsomes (0.053–0.213 mg/mL) and NADP (500 μM). Oxidation was not observed.

RAT TESTICULAR MICROSOMES

Preparation

These were prepared by the method of Al-Hamrouni *et al.*[24] except that the microsomal pellet was suspended in phosphate buffer (50 mM, pH 7.45) and homogenised before distribution and storage at −80 °C.

Assay for Reduction of Androstenedione

pH-Dependence

The method described for human testes microsomes was used with 0.5 μM substrate and 0.15 mg mL^{-1} protein. The results are shown in Figure 3. At low pH (pH 4.2 and pH 4.9) the conversion was nil, a result that was not unexpected since at these pH values, the concentration of reduced cofactor is low, so that reduction of androstenedione would not occur. From pH 4.9–6.5 the reduction of androstenedione

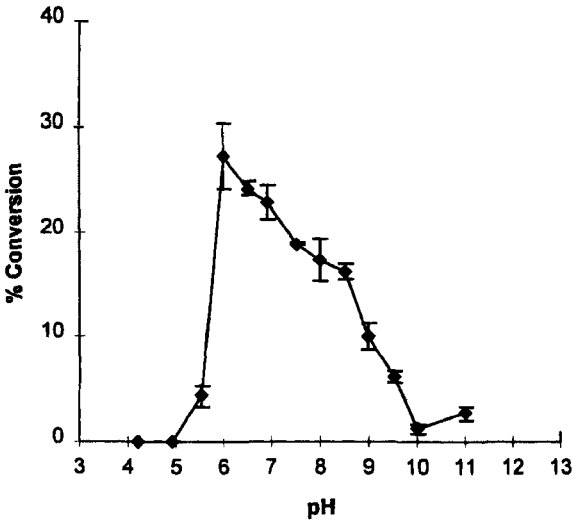


FIGURE 3 pH-Dependence of the reduction of androstenedione (0.5 μM) to testosterone by 17β-HSD from rat testes microsomes. Data are presented as the mean of duplicate samples and error bars show the spread of individual values. The results of this experiment were confirmed by an identical experiment.

increased drastically as the concentration of NADPH in the medium increased. However, the maximum pH for NADPH stability was pH 7.5, whereas the maximum conversion of androstenedione occurred around pH 6.0. The conversion decreased from pH 6.5 to pH 11.0, more rapidly than the stability of NADPH in this pH range. From these data, it was concluded that the optimum pH for the reduction of androstenedione to testosterone by the 17β-HSD of rat testes microsomes was around pH 6.0.

K_m *Value*

The assay was performed in duplicate as previously described for human testes microsomes using a range of androstenedione concentrations (0.14–4.0 μM) and microsomes preparations (0.15–0.3 mg/mL) for 30 min, at 37 °C.

The results were plotted by Lineweaver-Burk, Hanes-Woolf and Eadie-Hofstee plots (not shown) and gave a K_m of 0.77 ± 0.26 μM. The overal V_{max} value was 0.056 ± 0.005 nmol min^{-1} · mg $protein^{-1}$.

Inhibition Studies

The method previously described for human testes microsomes was used at substrate concentrations of 0.25 μM or 0.50 μM. The results are summarised in Table II and are the means of duplicate determinations (and individual values) or the mean of *n* determinations (± sd).

Assay for Oxidation of Testosterone

pH-Dependency

The method described for human testicular microsomes was followed with 0.25 μM substrate and 0.2 mg mL^{-1} protein. The results are shown in Figure 4 and demonstrate that the optimum pH for the reaction is around pH 9.5

K_m *Value*

Rat testicular microsomal preparation (0.46 mg/ml) was incubated in duplicate with a range of testosterone concentration (0.25–5.0 μM) under conditions previously described.

TABLE II Inhibitors of rat testes microsomal 17β-HSD for the reduction of androstenedione at pH 7.45

	Compounds	*% Inhibition*
1	Tamoxifen	71.9*
2	Clomiphene	63.6*
3		24.8*
4		19.1*
5	Phenyl-p-benzoquinone	96.2*
6	Diphenyl-p-benzoquinone	44.8*
7	2-Phenylhydroquinone	88.2*
8	Genistein	42.6
9	Biochanin A	63.0
10	6-Hydroxyflavone	40.6
11	7-Hydroxyflavone	33.9
12	Chrysin	nd
13	Apigenin	0
14	Baicalein	85.5
15	Fisetin	4.8
16	Morin	0
17	Myricetin	40.2
18	Naringenin	20.9

*Compounds at 200 μM, androstenedione at 0.25 μM or 0.5 μM; nd = not determined. The values are the means of duplicate samples.

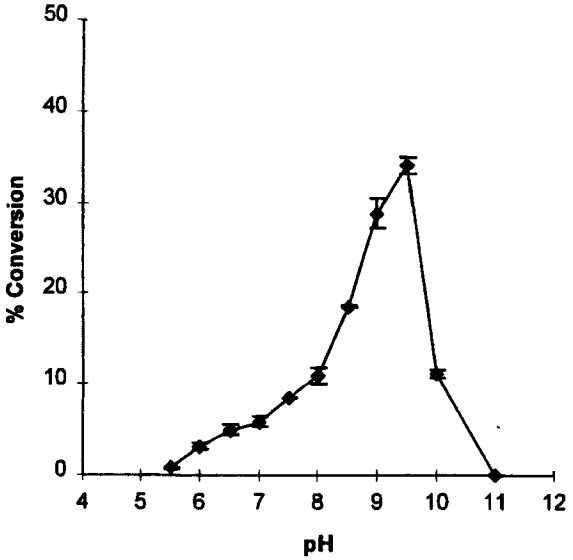


FIGURE 4 pH-Dependence of the oxidation of testosterone (0.25 μM) by 17β-HSD of rat testes microsomes. Data are presented as the mean of duplicate samples and error bars show the spread of individual values.

The K_m and V_{max} were 6.8 ± 2.1 μM and 0.064 ± 0.017 nmol $min^{-1} \cdot$ mg protein^{-1}.

Equilibrium for the Interconversion of Androstenedione/Testosterone

The method described for the human testicular microsomal preparation was followed except that a substrate concentration of (0.25 μM) and rat testicular microsomes (0.3 mg/mL) were used.

The results showed that the reduction reaction was favoured ($16.05 \pm 0.5\%$, $n = 4$) over the oxidation reaction ($12.3 \pm 0.15\%$, $n = 4$) under these conditions.

Reduction of Oestrone

The method described for human testes microsomes was followed except that oestrone (0.5 μM) was incubated with rat testes microsomes (0.03–0.46 mg/mL) at pH 7.45 for 30 min.

K_m Value

The K_m was determined by incubating rat testicular microsomes (0.185 mg/mL) with a range of oestrone concentrations (0.25–5.0 μM) at pH 7.45 for 30 min.

The K_m and V_{max} determined from the Lineweaver-Burk, the Eadie-Hofstee and the Hanes-Woolf plots were 3.3 ± 0.9 μM and 0.090 ± 0.018 nmol $\cdot$ min $^{-1} \cdot$ mg^{-1} respectively.

RESULTS AND DISCUSSION

Human Testes Microsomal 17β-HSD

The kinetic parameters, K_m and V_{max}, for the reduction of androstenedione were 0.28 ± 0.06 μM and 0.072 ± 0.014 nmol $\cdot$ min^{-1} mg^{-1} protein respectively. The K_m value was comparable with that obtained by Tunn *et al.*[25] for normal prostate ($K_m = 0.429$ μM) but not that for a crude preparation of human testes ($K_m = 1.0$ mM)[26] The pH-dependence for the reaction exhibited a maximum at pH 6.5 (Figure 1).

The reverse reaction, oxidation of testosterone to androstenedione, had K_m and V_{max} values of 17.2 ± 2.4 μM and 0.322 ± 0.053 nmol $\cdot$ min^{-1} mg^{-1} respectively. The K_m values is greater than that reported by Tunn *et al.*[25] with normal prostate homogenate ($K_m = 3.0$ μM). The pH-dependence for the reaction showed an optimum at pH 7.0 which is slightly higher than that for the reductive step (pH 6.5).

The K_m/V_{max} ratios for reduction of androstenedione and oxidation of testosterone were 3.88 and 53.4 respectively indicating that the reductive reaction was predominant. This was confirmed by a comparison of the two reactions simultaneously using ^{3}H-androstenedione and ^{14}C-testosterone at the same concentration and the appropriate coenzymes at pH 7.45 when the reduction reaction (21.3% conversion) was favoured over the oxidation reaction (14.8%).

The ability of the enzyme to catalyse the reduction/oxidation of oestrogens was examined. The enzyme was only capable of reducing oestrone to oestrodiol at a reasonable rate ($K_m = 11.6 \pm 12\,\mu M$, $V_{max} = 0.231 \pm 0.019\,nmol \cdot min^{-1}\,mg^{-1}$) and showed little activity with oestradiol as substrate.

The 17β-HSD type 3 present in human testes microsomes[16] specifically converts androstenedione to testosterone[14] with a low level of reduction of oestrone to oestradiol.[12] This data is in accord with the results obtained in this work where the ratio K_m/V_{max} for conversion of androstenedione, testosterone and oestrone was 3.88, 53.4 and 50.2 respectively and further confirmed by a study of the equilibrium androstenedione $\Leftrightarrow$ testosterone by differential substrate labelling. The optimum pH reported by Luu-The *et al.*[12] for reduction of androstenedione by recombinant 17β-HSD type 3 (pH 5.0) is slightly lower than that reported here (pH 6.5) but the former value was obtained using intact cells and pH 5.0 is that of the medium which may differ from the intracellular pH.

Taken together the results reported here indicate that the human testes microsomal 17β-HSD studied is the type 3 isoenzyme.

A range of compounds were examined at 200 μM as inhibitors of human testes microsomal 17β-HSD in the reduction of androstenedione. The results (% inhibition) for the active compounds are summarised in Table I as well as the IC_{50} values for the more potent compounds. The most potent compounds were benzoquinone derivatives diphenyl-p-benzoquinone (2.7 μM), and phenyl-p-benzoquinone (5.7 μM), and the flavones 7-hydroxyflavone (9.0 μM), baicalein (9.3 μM) and isoflavone biochanin A (10.8 μM).

Structure-activity relationships within the flavones showed that further mono- or dihydroxylation of 6-hydroxyflavone ($IC_{50} = 16.4\,\mu M$) or 7-hydroxyflavone (9.0 μM) in either one or both aryl rings maintained potency (baicalein, 9.3 μM; apigenin, 21.3 μM) but further hydroxylation reduced activity (fisetin, 32.4 μM; myricetin, 100.5 μM). Reduction of the 2,3-double bond of apigenin (21.3 μM) gave naringenin (16.4 μM) with similar activity. The isoflavones genistein (30.3 μM) and its 4′-methylether (biochanin A, 10.8 μM) were comparable in activity to the flavone analogue, apigenin (21.3 μM).

Rat Testes Microsomal 17β-HSD

The kinetic parameters, K_m and V_{max}, for the reduction of androstenedione were $0.77 \pm 0.26\,\mu M$ and $0.056 \pm 0.005\,nmol \cdot min^{-1}\,mg^{-1}$ protein respectively showing a nearly 3-fold higher K_m value than the corresponding human tissue. The pH-dependence for this reaction exhibited a maximum at pH 6.0 which was similar to that for the human tissue (pH 6.5) but the curves for the two tissues (Figures 1 and 3) were not identical over the higher pH 8–10 range. The kinetic parameters, K_m and V_{max} for oxidation of testosterone to androstenedione were $6.8 \pm 2.1\,\mu M$ and $0.064 \pm 0.017\,nmol\,min^{-1}\,mg^{-1}$ protein respectively. The K_m/V_{max} ratio for the reduction and oxidation steps were 13.75 and 106.25 respectively indicating that the reduction step was dominant, a view confirmed using the differential labelling technique previously described where the reductive reaction (16.05% conversion) was preferred over the oxidative reaction (12.3%).

Oestrone was a substrate for the enzyme and the K_m and V_{max} values obtained were $3.3 \pm 0.9\,\mu M$ and $0.09 \pm 0.018\,nmol \cdot min^{-1}\,mg^{-1}$ protein. Thus although the enzyme's affinity for oestrone was less than that for androstenedione ($K_m = 0.77 \pm 0.26$ (M) the V_{max} values (0.056 and $0.09\,nmol \cdot min^{-1}\,mg^{-1}$) protein were similar. On the basis of the K_m/V_{max} ratio for androstenedione and oestrone the enzyme is 3-fold more reactive towards androstenedione.

Comparison of the K_m/V_{max} ratios with human and rat testes microsomes for reduction of androstenedione (3.9 and 13.75 respectively), oxidation of testosterone (53.4 and 106.25) and

reduction of oestrone (50.2 and 36.7) then differences are seen between the relative specificities of the two enzymes, especially between reduction of androstenedione and oestrone. A clear cut difference between the tissues was seen in the respective pH optimum for oxidation of testosterone (pH 9.5 for rat testes, pH 7.0 for human testes) (Figures 4 and 2).

The results of the screen with rat enzyme (Table II), with compounds at 200 μM and a lower substrate concentration (0.25 μM) used than for the human enzyme (0.5 μM) so as to increase potency, showed that the compounds which were active against the human enzyme were, with two exceptions, also inhibitors of the rat enzyme but to a lesser degree. The most potent inhibitors were the benzoquinone derivatives phenyl-p-benzoquinone (96.2%) and 2-phenylhydroquinone (88.2%) and the flavone baicalein (85.5%).

In conclusion, it seems unlikely that the rat testes microsomal 17β-HSD and the human testes microsomal type 3 isoform are sufficiently similar in their behaviour to inhibitors for the use of the rat testes enzyme to be recommended as a model system for the screening of inhibitors intended to target the human 17β-HSD type 3 isozyme.

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