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# Some Coumarins and Triphenylethene Derivatives as Inhibitors of Human Testes Microsomal 17β-hydroxysteroid Dehydrogenase (17β-HSD Type 3): Further Studies with Tamoxifen on the Rat Testes Microsomal Enzyme

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**The 7-hydroxycoumarins, umbelliferone and 4-methylumbelliferone ($IC_{50}$ = 1.4 and 1.9 μM, respectively) were potent inhibitors of human testes microsomal 17β-HSD (type 3) enzyme whereas 7-methoxycoumarin, 4-hydroxycoumarin and 7-ethoxycoumarin had little or no inhibitory activity. Analogues of the weak inhibitory triphenylethenes tamoxifen and clomiphene but lacking the basic substituent, were weak inhibitors of the human microsomal enzyme. Inhibitory activity was improved by replacement of the triphenylethene structure with a triphenylmethyl (17, 52.6% inhibition) or phenylpropyl (16, 94.8%, $IC_{50}$ = 42.1 μM) skeleton. Further studies on tamoxifen using rat testes microsomal 17β-HSD showed that the inhibition was time-dependent and irreversible but not specifically mechanism-based.**

*Keywords*: 17β-Hydroxysteroid dehydrogenase (17β-HSD); Tamoxiphen; Clomiphene; Umbelliferone; Coumarins

## 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 and 5α-steroid reductase inhibitors as well as GnRH agonists/antagonists). 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) (Fig. 1).

17β-HSD exists in multi isoenzyme forms and types 1–9 have been identified in various tissues species using molecular biological tools.[1] 17β-HSD type 1 human placenta soluble (cytosolic) enzyme[2–5] shows a high degree of specificity for C18 steroids (oestrone, $E_1$; oestradiol, $E_2$) since C19 (androstenedione, testosterone (T)) and C21 (dihydroprogesterone) steroids are bound as substrates or inhibitors with much lower affinity than oestradiol.[6,7] The type 1 soluble enzyme from placenta was about equally reactive with $E_1$ and $E_2$ when activity was assessed on subcellular fractions,[8] 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 human placental microsomes[11] recognises C18, C19 and C21 steroids as substrates with comparable affinities, possesses 20α-HSD activity, and preferentially catalyses the oxidative step.[11–13] The 17β-HSD type 3 from human testes microsomes 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, i.e. type 2[15] although this was unconfirmed by Casey *et al.*[16]

17β-HSD is central to the conversion of weak androgen to its potent form and inhibitors of testicular 17β-HSD type 3 could provide novel agents for the treatment of hormone-dependent prostatic cancer (see review 17). In a screening programme for inhibitors of human testis

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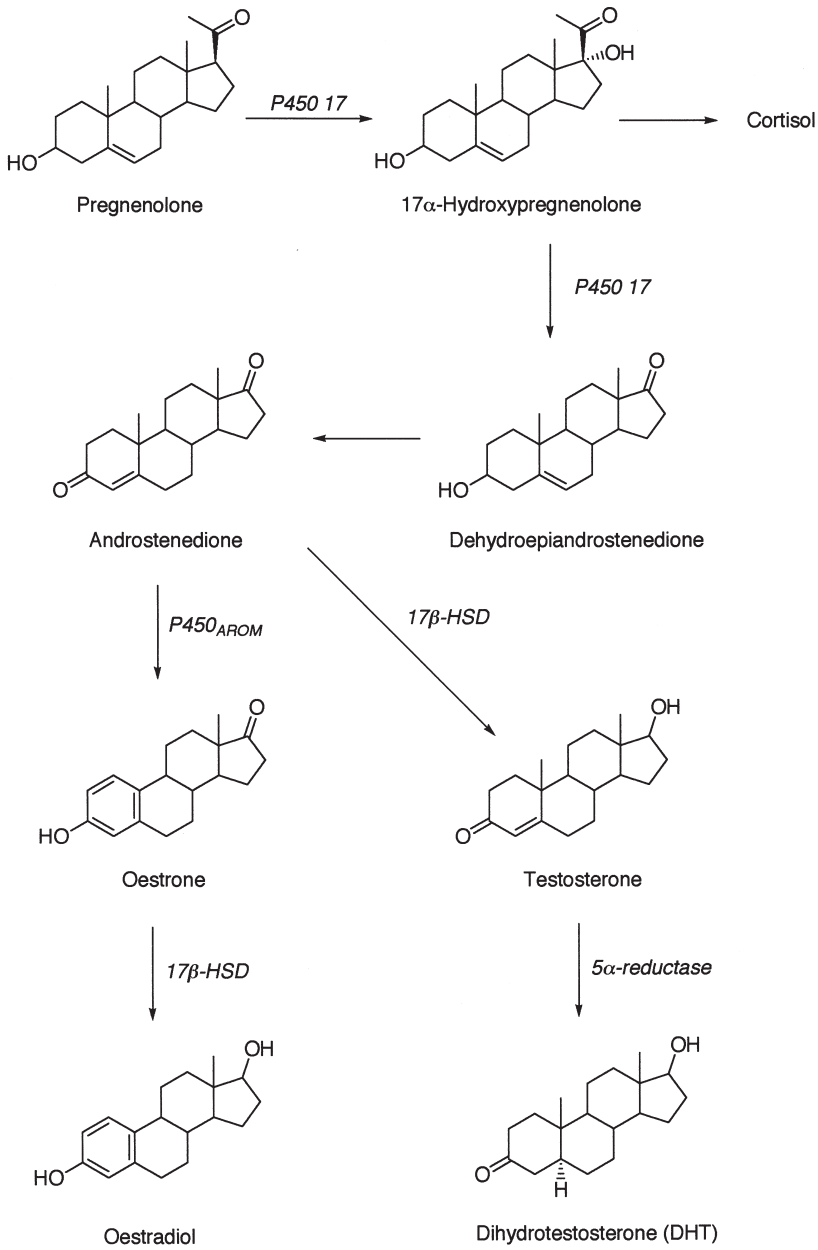



FIGURE 1 Androgen steroidogenesis pathway.

17β-hydroxysteroid dehydrogenase (17β-HSD type 3) 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 that reported for recombinant Type 3 17β-HSD from the same source.[18]

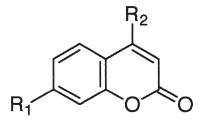
We have recently described some flavones and tamoxifen and other triphenylethenes as inhibitors of the human enzyme.[18] Here this study is extended to further triphenylethenes and coumarins on the human enzyme and a more detailed examination of the inhibition of tamoxifen using the more readily available rat enzyme.

## MATERIALS AND METHODS

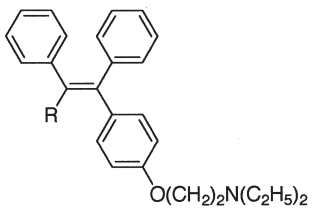
[1,2,6,7-$^3$H]-Androstenedione (86.4 Ci/mmol—37 MBq/ml) [1β-$^3$H]-androstenedione (24.3 Ci/mmol) and [4-$^{14}$C]-testosterone (53.9 Ci/mmol—0.74 MBq/ml) were purchases from NEN-Dupont UK (Stevenage, Herts). Androstenedione and testosterone were from Sigma Chemical Co. (Poole, UK). D-Glucose-6-phosphate dehydrogenase (G6PD); (suspension in ammonium phosphate) was obtained from Boehringer Mannheim (Mannheim, Germany). 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, UK) were used.

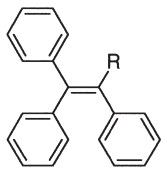
Tamoxifen (**7**), clomiphene (**8**) and the coumarins (**1**–**6**) were purchased from Sigma Chemical Co. (Poole, UK). Compounds (**9**), (**10**), (**11**), (**12**), (**16**) and (**17**) have been previously described by us.[19] Compounds (**13**), (**14**) and (**15**) were prepared according to Scheme 1 commencing with the known compound (**10**).[19]



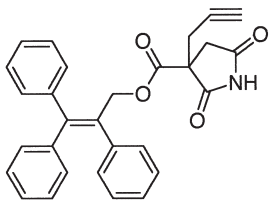
(**1**): $R_1 = R_2 = H$
(**2**): $R_1 = OH$, $R_2 = H$
(**3**): $R_1 = OH$, $R_2 = CH_3$
(**4**): $R_1 = H$, $R_2 = OH$
(**5**): $R_1 = OCH_3$, $R_2 = H$
(**6**): $R_1 = OC_2H_5$, $R_2 = H$



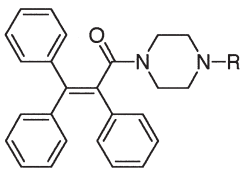
(**7**): Tamoxifen; $R = C_2H_5$
(**8**): Clomiphene; R = Cl



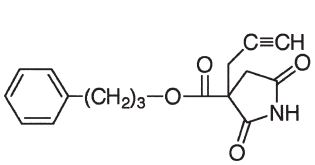
(**9**): R = Br
(**10**): $R = CO_2H$
(**11**): $R = CH_2OH$



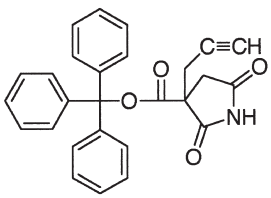
(**12**)



(**13**): $R = COOC_2H_5$
(**14**): $R = CH_2COOC_2H_5$
(**15**): $R = CH_2CH_2OH$



(**16**)



(**17**)

### Ethyl 4-(1-keto-2,3,3-triphenylprop-1-ene)-1-piperazine Carboxylate (13)

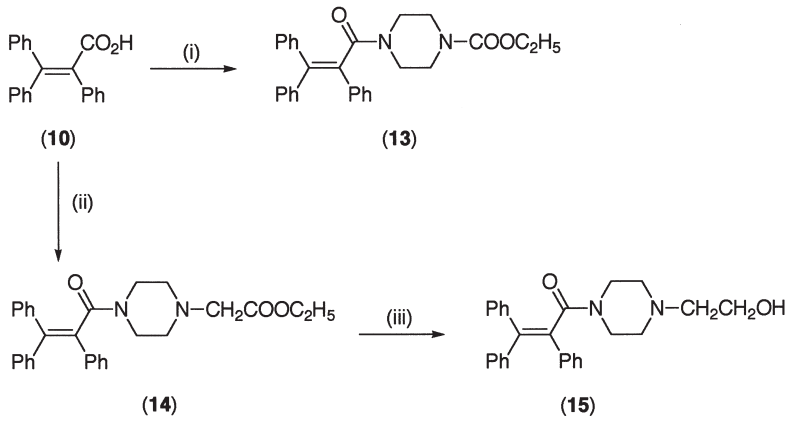
To a suspension of the acid (**10**) (1 g, 3.3 mmol) in benzene (25 ml) (CARE-CARCINOGEN) was added thionyl chloride (1.12, 0.73 ml, 10 mmol). The mixture was stirred and refluxed for 1 h and then evaporated. The residue was redissolved in benzene (25 ml) and ethyl 1-piperazine-carboxylate added at a temperature of 4°C and the mixture then stirred overnight at room temperature. The solvent was removed to give a crusty yellow solid (1.54 g). Recrystallisation from ethanol gave a white crystalline solid (0.8 g, 55% needles), m.p. 158.0–158.5°C, $R_f = 0.6$ (chloroform/methanol), (9:1). (Found: C, 76.3; H, 6.3; N, 6.4. $C_{28}H_{28}N_2O_3$ requires C, 76.35; H, 6.41; N, 6.36%). $\nu_{max}$ (KBr)$cm^{-1}$ 1700 (C = O ester), 1635 and 1610 (C = O amide); $\delta_H$ 1.32 (3H, t, J 7 Hz,$-CO_2CH_2CH_3$), 2.5–3.8 (8H, unresolved, piperazine), 4.23 (2H, q, J7 Hz,$-CO_2CH_2CH_3$), 7.00–7.55 (15H, m, aromatic).

### Ethyl 4-(1-keto-2,3,3-triphenylprop-2-ene)-1-piperazine Ethanoate (14)

To a suspension of acid (**10**) (1 g, 6.3 mmol) in benzene (50 ml) was added an excess of oxalyl chloride (2.2 ml, 25 mmol). The reaction was initiated with one drop of DMF and then stirred at room temperature 1 h. The solvent and oxalyl chloride were removed and the residue redissolved in benzene (50 ml). *N*-Carboethyoxymethylpiperazine (2.2 g, 12.6 mmol) was added dropwise to the solution at 4°C and the mixture stirred overnight at room temperature. The solvent was removed to give a pale orange solid which was recrystallised from ether/petroleum ether 40–60° (2:1) to give a white crystalline solid (1.62 g, 56%). M.p. 105–106°C (Found: C, 76.6; H, 6.7; N, 6.0. $C_{29}H_{30}N_2O_3$ requires C, 76.63; H, 6.65; N, 5.98%). $\nu_{max}$ (KBr)/$cm^{-1}$ 1750 (C = O ester), 1630 and 1610 (C = O amide); $\delta_H$ 1.25 (3H, t, J7 Hz, $-CO_2CH_2CH_3$), 1.90–2.50 (4H, unresolved, $-CH_2N(CH_2)-$ piperazine), 3.05 (2H, s, $-NCH_2-$), 3.30–3.90 (4H, unresolved, $-CH_2N(CH_2)CO-$, piperazine), 4.17 (2H, q, J7 Hz, $-CO_2CH_2CH_3$), 6.90–7.40 (15H, m, aromatic).

### 4-(1-keto-2,3,3-triphenylprop-2-ene)-1-piperazine Ethanol (15)

The ester (**14**) (0.73 g, 1.6 mmol) was added to an excess of lithium borohydride (2.0 M solution in THF (1.8 ml, 3.6 mmol) in THF (20 ml). The reaction was stirred overnight and the excess reducing agent destroyed with dilute hydrochloric acid (1 M). The solvent was removed and the residue dissolved in dichloromethane (25 ml), washed with a saturated sodium bicarbonate solution and then with water, dried ($MgSO_4$) and evaporated. The crude solid




SCHEME 1 Synthesis of some 4-(1-keto-1,2,2-triphenylethene)-1-piperazine derivatives. *Reagents and conditions*: (i) $C_6H_6$, $SO_2Cl_2$, reflux, 1 h; $C_6H_6$, ethyl 1-piperazine carboxylate, RT, 24 h (ii) $C_6H_6$, $(COCl)_2$, DMF, RT, 1 h; $C_6H_6$, *N*-carboethoxymethyl piperazine, RT, 24 h (iii) $LiBH_4$, THF, RT, 24 h; $H_3O^+$.

(0.65 g) was crystallised from ethanol to yield a white crystalline solid (0.45 g, 68%) m.p. 153.5–154.5°C Rf = 0.45 (ethyl acetate/methanol, 2:1). (Found: C, 78.5; H, 7.1; N, 6.8. $C_{27}H_{28}N_2O_2$ requires C, 78.61; H, 6.84; N, 6.79%) $\nu_{max}$ (KBr)/$cm^{-1}$ 3450 (OH), 1630 and 1620 (C = O amide); δH 1.80–2.50 (7H, unresolved, OH, $CH_2$–N($CH_2$)–, piperazine), 3.30–3.80 (6H, unresolved, –$CH_2$N($CH_2$)CO–, piperazine ring), 6.9–7.5 (15H, m, aromatic).

### Preparation of Human Testicular Microsomes

The testes, from a 65 years old patient undergoing orchidectomy with prostatic cancer, were cooled, mixed with phosphate buffer (50 mM, pH 7.45) containing sucrose (0.25 M), (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.*[20] The microsomal pellet was suspended in phosphate buffer (50 mM, pH 7.45), homogenised and stored at −80°C.

### Assay for Reduction of Androstenedione to Testosterone

A solution of [1,2,6,7-$^3$H]-androstenedione 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, glucose-6-phosphate 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 μl 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.

### $K_m$ 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 $\nu_{max}$ 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] androstenedione and androstenedione (0.5 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 Inhibition of human testis microsomal 17β-HSD type 3 by some coumarins and triphenylethene derivates

| Compounds* | % Inhibition† | $IC_{50}$‡ (μM) |
|---|---|---|
| *Coumarins* | | |
| Coumarin (**1**) | 5.7 | ND |
| Umbelliferone (**2**) | 100 | 1.4 |
| 4-Methylumbelliferone (**3**) | 100 | 0.91 |
| 4-Hydroxycoumarin (**4**) | 13.1 | ND |
| 7-Methoxycoumarin (**5**) | 54.9 | ND |
| 7-Ethoxycoumarin (**6**) | 0.0 | ND |
| *Triphenylethenes* | | |
| Tamoxifen (**7**) | 100¶ | 98.1¶ |
| Clomiphene (**8**) | 94.4¶ | 76.2¶ |
| (**9**) | 0.0 | ND |
| (**10**) | 0.0 | ND |
| (**11**) | 13.6 | ND |
| (**12**) | 86.7¶ | 9.15¶ |
| (**13**) | 49.6 | ND |
| (**14**) | 52.1¶ | ND |
| (**15**) | 34.6 | ND |
| (**16**) | 94.8 | 42.1 |
| (**17**) | 52.7 | ND |

*200 μM. †0.5 μM Androstenedione. ‡2.0 μM Androstenedione. ND, not done. Mean of duplicate samples where spread was <5%. ¶Ref. 18.

Table I, are the mean percentage 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.

### Preparation of Rat Testicular Microsomes

These were prepared by the method of Al-Hamrouni *et al.*[20] except that the microsomal pellet was suspended in phosphate buffer (50 mM, pH 7.45) and homogenised before distribution and storage at − 80°C.

### Screening of Tamoxifen as Inhibitor of Rat Testicular Microsomal 17β-HSD

An aliquot (10 μl, 0.5 μM final concentration) of [1β-$^3$H]-androst-4-ene-3, 17-dione in propylene glycol was incubated (in triplicate) at 37°C with rat testicular microsomes (0.036 mg/ml final concentration, 10 μl), NADPH generating system (50 μl) tamoxifen (10 μl, 100 μM final concentration) in ethanol and made up to 0.5 ml with phosphate buffer (50 mM, pH 7.4, 430 μl). In a control the inhibitor solution was replaced by ethanol (10 μl). After 15 min the reaction was terminated by addition of diethyl ether (3 ml), [14-$^{14}$C] testosterone (10 μl, approx. 3000 cpm) added and the mixture processed in a similar manner to that for human testicular microsomes. The results are given in Table II.

TABLE II Studies on the inhibition of rat testicular microsomal 17β-HSD by tamoxifen

| Procedure | % Inhibition* |
|---|---|
| Screening | 87 |
| Preincubation with enzyme | |
| Charcoal removal of inhibitor | 87.6 |
| *Validation*: | |
| *Preincubation* | |
| (a) in the presence of enzyme | 90.4 |
| (b) in the absence of enzyme | 1.6 |
| *Androstenedione protection*: | |
| (a) presence of substrate | 73.4 |
| (b) absence of substrate | 88.3 |
| *NADPH-dependence*: | |
| (a) presence of NADPH | 86.9 |
| (b) absence of NADPH | 78.4 |
| *Dialysis*: | |
| (a) pre-dialysis† | 72.0 |
| (b) post dialysis† | 64.5‡ |

*Tamoxifen = 100 μM, androstenedione = 0.5 μM. †Protein remaining = 57% in control and incubate after dialysis. ‡Androstenedione = 1 μM.

### Screening of Tamoxifen as an Irreversible Inhibitor of Rat Testicular Microsomal 17β-HSD

Rat testicular microsomes (20 μl; 0.072 mg/ml) tamoxifen (100 μM final concentration, 10 μl in ethanol) and NADPH generating system (50 μl) were made up to 0.5 ml with phosphate buffer (50 mM, pH 7.4, 420 μl) and pre-incubated at 30°C in a shaking water bath. After incubation for 10 min activated charcoal (250 μl, 1% suspension) was added to each tube and the mixture vortexed for 20 s. In a control determination the inhibitor solution was replaced by ethanol (10 μl). The tubes were then allowed to stand on ice for 20 min to ensure total adsorption of the unreacted inhibitors onto the charcoal, followed by centrifugation at 1500 rpm (1000*g*) for 15 min. Aliquots (440 μl) of the supernatant, in triplicate, were added to tubes containing NADPH generating system (50 μl) and the mixtures allowed to warm to 37°C for 2 min. [1β-$^3$H]-androstenedione (10 μl, 0.5 μM final concentration) was added to each tube and the mixtures incubated for 15 min. The assays were worked up in the usual manner as described previously. The results are given in Table II.

### Validation of the Pre-incubation Technique for Detection of Irreversible Inhibition

Here, the separation method used was validated to ensure total adsorption of the unreacted inhibitor onto the charcoal during the assay. In a control determination the inhibitor solution was replaced by ethanol (10 μl). Pre-incubation was carried out in phosphate buffer (50 mM, pH 7.4) containing tamoxifen (100 μM, 10 μl) in ethanol and NADPH generating system (50 μl), but in the absence of the rat testicular microsomes, at 37°C for 10 min

followed by addition of 1% charcoal (250 μl) and vortexing for 20 s. The mixtures were allowed to stand on ice for 20 min and centrifuged at 1500 rpm (1000*g*) for 15 min. Aliquots (420 μl) of the supernatant in triplicate were added to tubes containing rat microsomal protein (20 μl, 0.072 mg/ml) and the NADPH generating system (50 μl). The mixtures were warmed to 37°C for 2 min and the reaction was initiated by addition of the [1β-$^3$H]-androstenedione (0.5 μM, 10 μl). After 15 min the reaction was stopped and the experiment was continued as previously described. The results are shown in Table II.

### Androstenedione Protection

A solution of tamoxifen (100 μM, 10 μl) in ethanol, microsomal protein (20 μl, 0.072 mg/ml) and NADPH generating system (50 μl) in the presence and absence (replaced by phosphate buffer) of [1β-$^3$H]-androstenedione (0.5 μM, 10 μl) was incubated in a total volume of 0.5 ml for 10 min at 30°C. In a control determination the inhibitor solution was replaced by ethanol (10 μl). Activated charcoal (250 μl, 1% suspension) was added to each tube and then the method described above for screening of tamoxifen as an irreversible inhibitor was followed. The results are shown in Table II.

### NADPH-dependency

A solution of tamoxifen (100 μM, 10 μl in ethanol), microsomal protein (20 μl, 0.072 mg/ml) with and without (replaced by phosphate buffer) NADPH generating system (50 μl) made up with phosphate buffer (50 mM, pH 7.4) to a final incubation volume of 0.5 ml was incubated for 10 min at 30°C. In a control determination the inhibitor solution was replaced by ethanol (10 μl). Activated charcoal (250 μl, 1% suspension) was then added to each tube and the method then followed as described above for androstenedione protection. The results are shown in Table II.

### Dialysis of the Inhibited Enzyme

A solution of tamoxifen (100 μM, 60 μl) in ethanol was added (in triplicate) to tubes containing microsomal protein (0.132 mg/ml, 220 μl) and NADPH generating system (300 μl) and made up to 3.0 ml with phosphate buffer (50 mM, pH 7.4, 2420 μl). Control incubations contained ethanol without inhibitor. After incubation for 10 min at 30°C, the tubes were removed and placed on ice.

(a) Aliquots (430 μl), in triplicate, were removed and added to tubes containing NADPH generating system (50 μl). The mixture was warmed to 37°C for 2 min and the reaction was initiated by the addition of [1β-$^3$H]-androstenedione (1 μM, 20 μl). After 15 min incubation period the experiment was continued as previously described for screening of tamoxifen as an inhibitor. (b) Aliquots (2 ml) from the remaining incubation mixture were removed, transferred to dialysis tubing (pore size 2.4 nm; $M_r$ threshold = 12,000–14,000 and then dialysed against phosphate buffer (50 mM, pH 7.4) for a period of 8 h at 4°C. The dialysis reservoir was shaken every 2 h. After dialysis, aliquots (430 μl) from each tube, in triplicate, were removed and the remaining enzyme activity determined as for (a) above.

Determination of the protein content of the solution in the dialysis tube was carried out before and after the dialysis, using the Pierce BCA protein assay as previously described.

The results obtained are summarised in Table II and show that irreversible inhibition had occurred since activity was not regenerated on dialysis. However, a relatively high loss of protein (43%) occurred during dialysis of the control and inhibitor incubates.

### Kinetics of Inactivation

Tamoxifen (50–70 μM final concentration, 10 μl) was separately added (in triplicate) to tubes containing microsomal protein (0.072 mg/ml, 20 μl) and NADPH generating system (50 μl), which had been warmed to 30°C for 5 min, and made up to 0.5 ml with phosphate buffer (50 mM, pH 7.4). Control incubations contained ethanol without inhibitor. After incubation of each concentration for different time intervals at 30°C, activated charcoal (250 μl, 1% suspension) was added to each tube and then the method described above for androstenedione protection followed.

A plot of (% activity remaining) vs (pre-incubation time) is shown in Fig. 2. A plot of the data as (log % activity remaining) vs (pre-incubation time) is shown in Fig. 3.

## RESULTS AND DISCUSSION

In previous studies we have shown that human testes microsomal 17β-HSD has the characteristics of the recombinant type 3 isozyme from the same source.[18] We have previously described some flavones/isoflavones and the triphenylethenes tamoxifen and clomiphene and synthetic analogues (**12**) and (**14**) as inhibitors of the microsomal enzyme.[18] Further work on coumarins and synthetic triphenylethenes and related compounds as inhibitors is described here. The 7-hydroxycoumarins, umbelliferone (**2**) and 4-methylumbelliferone (**3**), were potent inhibitors of the human testes

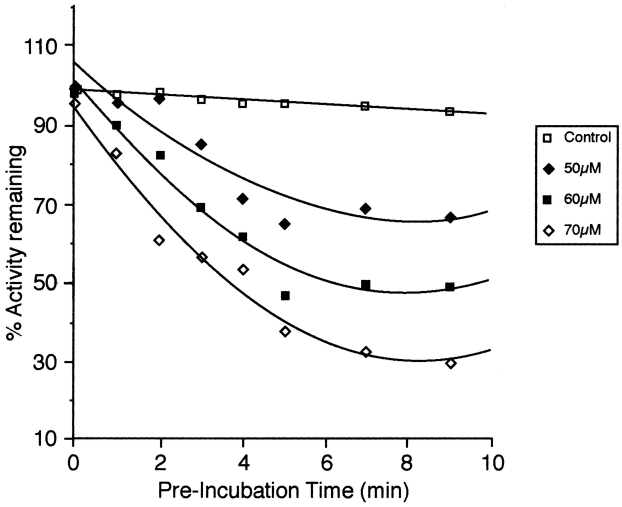



FIGURE 2 Preincubation and concentration dependency of inactivation of rat testicular microsomal 17β-HSD by tamoxifen (50–70 μM) with protein concentration 0.072 mg ml$^{-1}$. Points are the mean of two experiments, each with triplicate tubes.

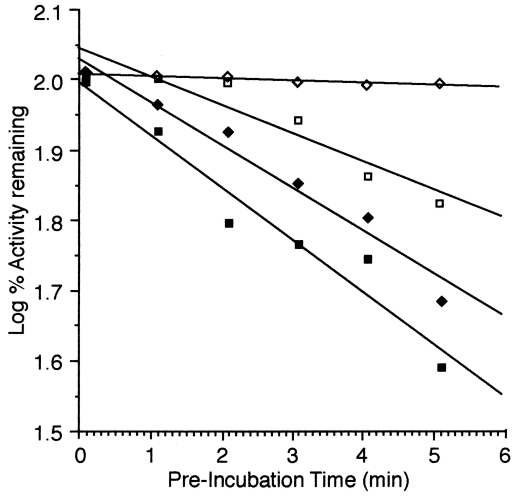



FIGURE 3 Log% 17-β HSD activity remaining vs pre-incubation time from Figure 2.

microsomal 17β-HSD (type 3) enzyme ($IC_{50}$ = 1.4 and 1.9 μM, respectively). 7-Methoxycoumarin (**5**) had weak inhibitory activity (54.9% inhibition) whereas coumarin (**1**), 4-hydroxycoumarin (**4**) and 7-ethoxycoumarin (**6**) were practically inert (Table I).

We have reported tamoxifen (**7**) and clomiphene (**8**), triphenylethenes with basic substituents, as potent inhibitors of the enzyme (100 and 94.4%, respectively). although this high activity was not borne out by their $IC_{50}$ values (98.1 and 76.2 μM, respectively). Here, other triphenylethenes from our chemical library were examined.

The triphenylethene ester (**12**) had been previously described[19] as a moderately potent inhibitor of the enzyme (86.7%, $IC_{50}$ 9.15 μM) but the $-CH_2OH$ (**11**), −COOH (**10**) and −Br (**9**) intermediates lacked activity. Similarly, modification of the weak inhibitor (**14**) possessing a 4-carboethoxymethyl piperazine side chain (52.1%) to the $-COOC_2H_5$ (**13**, 49.6%) and $-CH_2CH_2OH$ (**15**, 34.6%) did not improve inhibitory potency. However replacement of the triphenylethene structure in (**12**) with triphenylmethyl (**17**, 52.6%) or phenylpropyl (**16**, 94.8%, $IC_{50}$ = 42.1 μM) gave weak inhibitors.

Further studies were conducted on tamoxifen using the more readily available rat testes microsomal 17β-HSD. We have previously shown[18] that although there are some differences between the human and rat testes enzyme, the rat enzyme is suitable for screening inhibitors of 17β-HSD although inhibition is slightly less marked.

Tamoxifen, on preincubation with rat testicular microsomal enzyme followed by removal of the inhibitor on charcoal, showed irreversible inhibition (87.6%) of the rat testes enzyme (Table II) since activity was not regenerated on dialysis (predialysis 72.0% inhibition, postdialysis 64.5%) despite a 43% loss of protein in the dialysed control and inhibitor incubates. Attempts to confirm the inhibition as being irreversible using human male fibroblast cell cultures were unsuccessful (not shown).

Further experiments were conducted to determine whether tamoxifen was a mechanism-based inactivator. An androstenedione protection study with tamoxifen (100 μM) showed that inhibition decreased from 88.3% in the absence of the substrate to 73.4% in its presence in the incubation medium indicating that a small protection occurred (Table II). A NADPH study with tamoxifen showed that the inhibition was slightly decreased from 86.9% in the presence of the NADPH generating system to 78.4% in its absence (Table II). This difference is very small compared with that found for the propynyl 3-pyrrolidine-2,5-dione derivatives and the aromatase enzyme.[21] This may indicate that the irreversible inhibition noted is not mechanism-based.

Incubation of 17β-HSD enzyme with different concentrations of tamoxifen for various time intervals gave a time-dependent loss of enzyme activity characteristic of irreversible inhibition (Figs. 2 and 3). A plot[22] of the reciprocal of the inhibitor concentration versus inactivation half-life gave a linear curve but this did not cut the Y-axis as expected at T1/2 as observed previously by us for mechanism-based inhibition of aromatase by a series of propynyl 3-phenylpyrrolidine-2,5-diones.[21]

In summary, it has been shown that tamoxifen is a time-dependent and irreversible inhibitor of 17β-HSD. The inhibition does not appear to be mechanism-based but is time-dependent and there is the possibility that a slow-off tight-binding process is involved.

Tight binding inhibitors are potent and can show a time-dependency as they position themselves in

their optimum binding conformation at the active site. Tamoxifen is not a potent inhibitor by comparison as can be seen in Fig. 2 where at a 50 μM concentration only about 30% inhibition occurs. However, it is a bulky molecule and may require time to orientate itself in the active site. A more speculative explanation would be that there is a very small amount of "impurity" in tamoxifen which is the active species. Bulk tamoxifen consists of mainly *Z*-tamoxifen (i.e. trans positioning of two unsubstituted phenyl groups) with some *E*-tamoxifen[23] which is limited to 1% in the bulk drug by the British and US Pharmacopoeias. However *E*-tamoxifen does not constitute the active species in the bulk drug since *E*-tamoxifen is only a slightly better inhibitor of the enzyme than tamoxifen.[24] Further experiments are required to examine the proposition that an active species is involved.

It seems unlikely that chronic dosing with tamoxifen would effectively reduce 17β-HSD levels although it might produce a small cumulative effect on 17β-HSD levels. This effect would probably be insufficient to warrant the use of tamoxifen as an anti-prostatic cancer drug in view of its effect on the oestrogen receptor and the overall oestrogen-androgen balance.

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