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New Aromatic Esters of Progesterone as Antiandrogens

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The *in vivo* and *in vitro* antiandrogenic activity of four new progesterone derivatives: 4-bromo-17α-(*p*-fluorobenzoyloxy)-4-pregnene-3,20-dione 1,4-bromo-17α-(*p*-chlorobenzoyloxy)-4-pregnene-3,20-dione 2, 4-bromo-17α-(*p*-bromobenzoyloxy)-4-pregnene-3,20-dione 3 and 4-bromo-17α-(*p*-toluoyloxy)-4-pregnene-3, 20-dione 4 was determined. These compounds were evaluated as antiandrogens on gonadectomized hamster prostate and reduced the weight of the prostate glands in gonadectomized hamsters treated with testosterone 5 (T) or dihydrotestosterone 6 (DHT) in a similar manner to that of commercially available finasteride, thus indicating a potent *in vivo* effect. The *in vitro* studies showed that steroids 1–4 have a weak inhibitory activity on 5α-reductase with IC_{50} values of: 280 (1), 2.6 (2), 1.6 (3) and 114 μM (4). The presence of Cl and Br atoms in the C-17 benzoyloxy group tends to increase the inhibitory potency of the compounds.

The binding efficiency of the synthesized steroids 1–4 to the androgen receptor of the prostate gland is also evaluated. All compounds form a complex with the receptor and this explains the weight reduction of the seminal vesicles in the animals treated with DHT plus steroids 1–4.

Keywords: 5α-Reductase inhibition; Novel steroids; Prostate growth; Androgen receptor; Binding affinity

INTRODUCTION

Androgen antagonists offer a potentially useful treatment for androgen-mediated diseases such as prostate cancer, seborrhea, androgenic alopecia and benign prostatic hyperplasia.[1] The most important therapeutic application of antiandrogens is in the treatment of prostate cancer and benign prostatic hyperplasia. Although surgery represents the most accepted treatment for prostate cancer (about 400,000 prostatectomies are performed each year in the USA) there are several other modalities available for the treatment of these diseases.[2] Dihydrotestosterone **6** (DHT) the 5α-reductase metabolite of testosterone **5** (T) has been implicated as a causative factor in the progression of these diseases.[3–5] It has also been observed that DHT interacts more efficiently with the androgen receptors than testosterone. This fact indicates that the logical step in the treatment of these diseases should be the inhibition of the enzyme 5α-reductase[6] so as to block DHT-receptor complex formation.[6]

Two isozymes of 5α-reductase have been discovered; type 1 is present in most tissues in the human body where 5α-reductase is the dominant form in sebaceous glands. Type 2 5α-reductase isozyme predominates in genital tissue, including the prostate.[7]

The most extensively studied class of 5α-reductase inhibitors is the 4-azasteroids[8,9] which includes the drug finasteride. This compound is the first 5α-reductase inhibitor approved in the USA for the treatment of BPH. It has approximately a 100-fold greater affinity for type 2- 5α-reductase, than for the type 1 enzyme. In humans, finasteride decreases prostatic DHT levels by 70–90% and reduces prostate size, while T tissue levels remain constant.[10] The use of finasteride demonstrated a sustained improvement in the treatment of androgen dependent diseases and also reduces the prostate specific antigen (PSA) levels.[11]

The structure–activity relationship of a series of halopregnane derivatives determined in

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our laboratory indicated that a bromine atom at C-6 and an ester side chain at C-17 in the progesterone skeleton increased antiandrogenic activity.[4–8]

In another study, we evaluated the pharmacological effect of 4-halo (bromine, chlorine and fluorine atoms) as well as the presence of the 17α-benzoyloxy moiety in the progesterone skeleton.[12] The pharmacological data of this study indicated that steroidal derivatives having a C-17 benzoyloxy moiety and a bromine atom a C-4 showed a higher antiandrogenic activity. As previously determined the ester moiety at C-17 is a necessary requirement for the presence of a high antiandrogenic effect.[13,14] In view of the fact that 4-halo and 6-bromo compounds showed a high pharmacological activity, an attempt was made to synthesize compounds in which the bromine, chlorine or fluorine atoms were incorporated in the ester side chain of the progesterone skeleton having a bromine atom a C-4.[13] All derivatives having this structural characteristics inhibited very efficiently the conversion of T to DHT in hamster seminal vesicles.

In this paper we report the antiandrogenic effect of four compounds: 4-bromo-17-α-(*p*-fluorobenzoyloxy)-4-pregnen-3,20-dione (**1**); 4-bromo-17-α-(*p*-chlorobenzoyloxy)-4-pregnen-3,20-dione (**2**); 4-bromo-17-α-(p-bromobenzoyloxy)-4-pregnene-3,20-dione (**3**) and 4-bromo-17-α-(*p*-toluoyloxy)-4-pregnen-3,20-dione (**4**) (Figure 1) in gonadectomized hamster prostate.

EXPERIMENTAL

Chemical and Radioactive Material

Solvents were laboratory grade or better. (1, 2, 6, 7-^{3}H) Testosterone ([^{3}H] T specific activity: 95 Ci/mmol) and (1, 2, 4, 5, 6, 7- ^{3} H) dihydrotestosterone ([^{3}H]DHT specific activity 110 Ci/mmol), were provided by New England Nuclear Corp. (Boston, Ma). Testosterone (T) and 5α-dihydrotestosterone were supplied by Steraloids (Wilton, NH, U.S.A.). Sigma Chemical Co. (ST. Louis, Mo) supplied NADPH. Finasteride was obtained by extraction from Proscar (Merck, Sharp & Dohme). The tablets were crushed, extracted with chloroform and the solvent removed and the crude product purified by silica gel column chromatography.

The synthesis of steroids **1**–**4** is described eldewhere.[13]

Animals and Tissues

Adult male golden hamsters (150–200 g) were obtained from Metropolitan University-Xochimilco of Mexico. Gonadectomies were performed under light ether anesthesia 30 d before the experiments. The animals were sacrificed under ether anesthesia. This protocol was approved by the Institutional Care and Use Committee of the Metropolitan University of Mexico (UAM).

The prostate glands were immediately removed, blotted and weighed prior to their use. Tissues used in the metabolic experiment were homogenized with

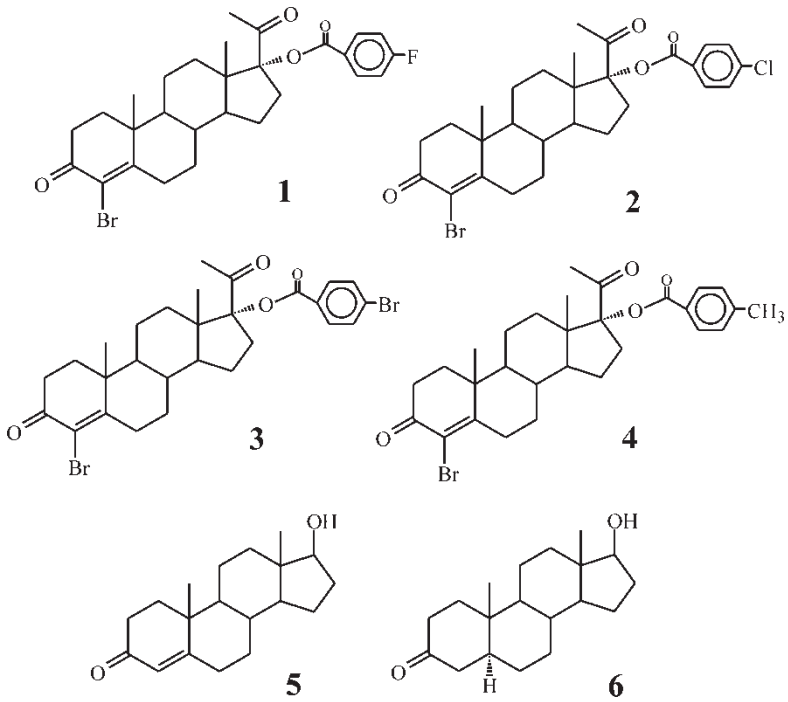

FIGURE 1 Steroid structures.

a tissue homogenizer (model 985-370; variable speed 5000–30,000 rpm, Biospec Products, Inc.).

In Vivo Experiments

The effect of the new steroids **1–4** on the prostate of male hamsters, which had been gonadectomized 30 days prior to the experiment, was determined on 14 groups of 4 animals/experiment, which were selected at random. The animals were kept in a room with controlled temperature (22°C) and light–dark periods of 12 h. Food and water were provided *ad libitum.*

Daily subcutaneous injections of 200 μg of the steroids **1–4** dissolved in 200 μl of sesame oil were administered for 6 days together with 200 μg of T or DHT. Four groups of animals were kept as control, one was injected with 200 μl of sesame oil, the second and third with 200 μg of testosterone or DHT and the fourth with T with finasteride for 6 days. After the treatment, the animals were sacrificed by ether anesthesia and the prostate gland was dissected and weighed. Four separate experiments were performed for each group of steroid treated animals. The results were analyzed using one-way analysis of variance with EPISTAT software (Table I).

In Vitro Experiments

Tissues were homogenized in 3 volumes of buffer TEMD (20 mM tris-HCl, 1.5 mM EDTA and 10 mM sodium molybdate) at pH 7.4 and 4°C with a tissue homogenizer. Homogenates were centrifuged at 140,000 × g for 60 min[14] in a SW 60 Ti rotor (Beckman instruments, palo Alto, CA). The pellets were separated, washed with 3 tissue volumes of medium A (20 mM sodium phosphate, pH 6.5 containing 0.32 M sucrose, 0.1 mM dithiothreitol Sigma-Aldrich, Inc) and centrifuged on two additional occasions at 440 × g at 0°C for 10 min.[15] The washed pellets were suspended in medium A and kept at −70°C. The suspension (6.8 mg protein/ml determined by the Bradford method[15] was used as source of 5α-reductase.

TABLE I Weight of prostate gland ± standard deviations from animals receiving for 6 days different s.c. treatments

Treatment (daily doses of 200 μg/200 μl)	Weight of prostate (mg)
Vehicle	44.9 ± 7.9
Testosterone (T)	87.6 ± 12.0
Dihydrotestosterone (DHT)	92.33 ± 17.5
T+F	69.3 ± 10.8
T+Steroid 1	72.58 ± 8.4
DHT+Steroid 1	75 ± 10.2
T+Steroid 2	75.8 ± 1.2
DHT+Steroid 2	70.9 ± 7.1
T+Steroid 3	77.6 ± 5.3
DHT+Steroid 3	68.8 ± 19.5
T+Steroid 4	70.9 ± 7.1
DHT+Steroid 4	60.4 ± 9.6

Significant differences ($p < 0.05$) were observed between T/DHT and control or T/DHT plus each one of different steroid treatments. Four groups of animals were kept as control, one was injected with 200 μl of sesame oil (Vehicle), the second and third with 200 μg of T or DHT and the fourth with T with finasteride (F) for 6 days (see experimental section).

Determination of 5α-reductase Activities

The enzyme 5α-reductase was assayed as previously described.[17] The reaction mixture (final volume of one ml) contained 1 mM dithiothreitol, sodium phosphate buffer (40 mM), 2 mM, NADPH, 2 nM [1,2,6,7-^{3}H]T. The reaction, in duplicate, was started when it was added to the enzymatic fraction (250 μg protein), incubated at 37°C for 60 min, and stopped by mixing with one ml of dichloromethane; this was considered as the end point. An icubation without tissue was used as a control. The dichloromethane fraction was separated and the extraction was repeated 4 more times. The combined extracts were evaporated under a nitrogen stream to dryness, suspended in 50 μl of methanol and spotted on HPTLC Keiselgel 60 F_{254} plates. T and DHT were used as carriers and the plate was developed in chloroform:acetone = 9:1. The plates were air-dried and the chromatography was repeated on 2 further occasions. The T standard was visualized under UV light (254 nm) and DHT was detected using phosphomolybdic acid reagent with under heating of the plate (DHT develops a classic dark blue color). DHT-containing areas were cut out and the strips were soaked 5 ml in Ultima Gold (Packard) and the radioactivity counted on the scintillation counter (Packard tri-carb 2100 TR).

When the assay was repeated, it was observed that the 5α-reductase enzyme from prostate hamster remained active for more than four months when stored at −70°C.

pH-dependent 5α-reductase Activity

To measure the pH-dependent 5α-reductase activity, we followed the method describe previously by Hirosumi *et al.*;[17] the assays were carried out in the reaction mixture (final volume of one ml) containing 1 mM DTT, 40 mM potasium phosphate buffers ranging from pH 4–9, 2 mM NADPH, 2–120 nM T and the prostatic enzyme fraction (approximately 250 μg). The reaction was carried out in duplicate at 37° C for 60 min and then the procedure described above followed. The kinetic parameters were analyzed according to the Lineweaver-Burk plot method.

Determination of IC_{50} Values for Steroids 1–4 on 5α-reductase from Gonadectomized Hamster Prostate

To calculate IC_{50} values (the concentration of **1–4** required to inhibit 5α-reductase activity by 50%), four series of tubes containing increasing concentrations of **1–4** (10^{-6}, 10^{-4} M) were incubated in duplicate, in the presence of 1 mM of dithiothreitol, 40 mM sodium phosphate buffer pH 7, 2 mM NADPH, 2 nM [1,2,6,7-^{3}H]T and 250 μg of protein from enzymatic fraction in a final volume of one ml. The reaction was carried out in duplicate at 37°C for 60 min. Addition of one ml of dichloromethane stopped the reaction, and the amount of DHT formed was determined as detailed above.

Binding Assay for the Androgen Receptor of Hamster Prostate

The saturation curve and determination of binding parameters for hamster prostate were determined by methods described previously.[14] The data were analyzed according to the Scatchard plot method.[18]

The cytosolic fraction obtained from the supernatant liquid of the prostate homogenate centrifuged at 140,000 × g as described above, was stored at −70°C. Prostatic cytosol (2.4 mg protein, determined by the Bradford method[16]) was incubated for 18 h in duplicate with 1–8 nM of [^{3}H]DHT in TEMD buffer containing 1 mM dithiothreitol in a final volume of 200 μl. Parallel sets of tubes containing identical concentrations of [^{3}H]DHT plus a 100-fold excess of radio inert steroid were incubated in the presence of cytosol for 18 h. After this time, 800 μl of dextran-coated charcoal in TEMD buffer was added and the mixture was incubated for 40 min at 4°C. To prepare the dextran-coated charcoal mixture, the dextran was agitated during 30 min before adding the charcoal to the mixture. The tubes were vortexed and immediately centrifuged at 800 g for 10 min; aliquot (200 μl) were subsequently submitted to radioactive counting. Specific binding was determined by subtracting the mean disintegration per minute (dpm) in the presence of excess unlabeled steroids from the mean dpm of the corresponding tubes containing only [^{3}H]DHT.

Competition Studies

For competition studies, tubes containing 3.15 nM of [^{3}H]DHT (Specific activity 110 Ci/mmol) plus a range of increasing concentrations (1×10^{-6}–1×10^{-3} M) of cold DHT and compounds **1–4** were prepared.[14]

Aliquots of 200 μl of prostatic cytosol (2.4 mg protein, determined by the Bradford method[16]) were added and incubated (in duplicate) for 18 h at 4°C in the tubes mentioned above. Then, 800 μl of dextran-coated charcoal in TEMD buffer (containing dithiotreitol) was added and the mixture incubated for 40 min at 4°C. The tubes were vortexed and immediately centrifuged at 800g for 10 min; aliquot (200 μl) were subsequently submitted to radioactive counting. The inhibition constant (K_i) of each compound was calculated according to the procedures described by Cheng and Prusoff.[19]

RESULTS

In Vivo Experiments

After castration, the weight of the male hamster prostates significantly decreased ($p < 0.005$) compared to that of the normal glands. Treatment with vehicle alone did not change this condition, whereas s.c. injections of 200 μg of T or DHT for 6 days significantly increased ($p < 0.005$) the weight of the prostates in castrated male hamsters (Table I).

When testosterone (T) and finasteride were injected together, the weight of the prostate decreased significantly ($p < 0.005$) as compared to testosterone-treated animals (entry 2, Table I). However, when the same experiment was carried out with compounds **1–4**, the weight of the prostate also decreased, thus showing a comparable antiandrogenic effect to that of finasteride. Daily injections for 6 days of DHT plus **1–4** significantly ($p < 0.005$) decreased the weight of the prostate as compared with DHT-treated animals (Table I), thus showing an antiandrogenic effect.

Since the weight of the prostate depends on the 5α-reduced androgens,[20] it was important to determine the effect of the new steroidal compounds (**1–4**) on the activity of 5α-reductase and their androgen receptor-binding affinity.

pH-dependent 5α-reductase Activities

Hamster prostatic 5α-reductase from the nuclear, mitochondrial and microsomal fraction of the prostate demonstrated a conversion of [^{3}H]T into [^{3}H]DHT in the presence of NADPH after one month of gonadectomy. The pH-profile of hamster 5α-reductase using different concentrations of T is shown in Figure 2. In the presence of 2 nM T, hamster 5α-reductase showed two maximum levels of conversion of T into DHT at pHs 6 and 7. With higher concentrations of T, the pH optimum was transferred to the more acidic range. In the presence of 60 nM of T, hamster 5α-reductase showed the maximum activity at around pH 5, whereas at 20 and 120 nM it was around 4.5. The conversion of [^{3}H]T to [^{3}H]5α-DHT at pH 5, 6 and 7 is shown in Table II, along with the calculated kinetic parameters

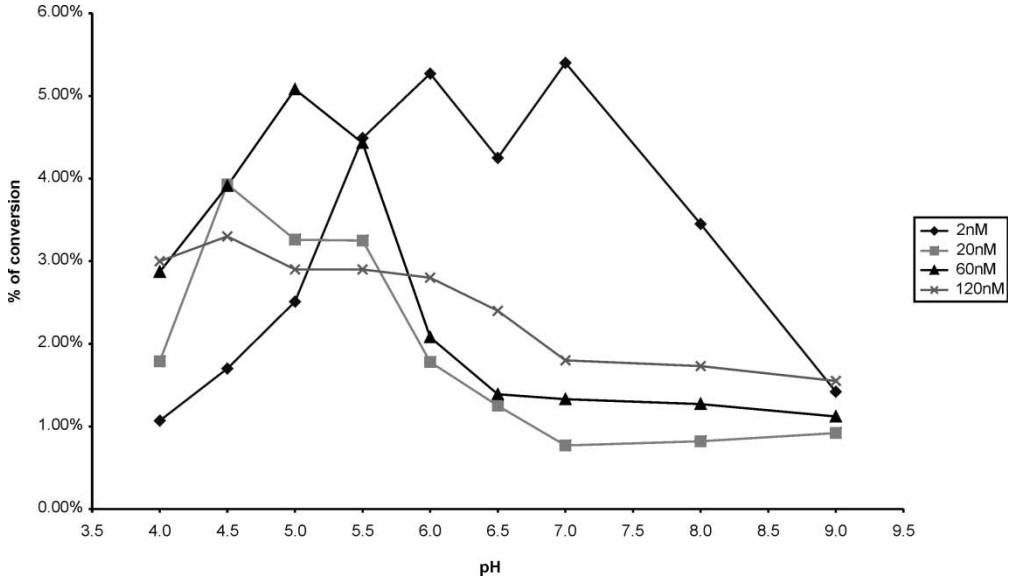

FIGURE 2 Effect of testosterone concentration on pH optima of hamster prostatic 5α-reductase activity. The reaction mixture contained 2, 20, 60, 120 nM [^{3}H]T and buffers ranging from pH 4–9 and were incubated in duplicate at 37°C for 60 min.

analyzed by the Lineweaver-Burk method. The conversion rate was greater at pH 7 than at pH 6 with 2 nM T, but opposite results were obtained with T at 20, 60 and 120 nM. This data indicates that the maximal velocity of this enzyme is higher at pH 4.5 (data no shown) and 5 than at pH 7, whereas the Km value is lower at pH 7 than at pH 5 and 6 (Table II).

Determination of IC_{50} Values for 1–4 for 5α-reductase of the Hamster Prostate

The concentrations of compounds **1**–**4** required to inhibit 5α-reductase activities by 50% (IC_{50}) was determined from the inhibition curves using different concentrations of the steroids and are shown in Table III.

The IC_{50} values obtained from the curves using compounds **1**–**4** and determined at pH of 7 were: **1** 280; **2** 2.6; **3** 1.6 and **4** 114 μM Steroid **3** showed the highest inhibitory potency of the studied compounds as indicated by its low IC_{50} value.

Binding Assay for the Androgen Receptor in Rat Prostatic Cytosol

Aliquots of cytosol (2.62 mg protein) obtained from prostate homogenates of gonadectomized hamsters were incubated with increasing concentrations of [^{3}H]DHT in order to obtain a typical saturation binding curve. The levels of non-specific bound hormone increased progressively with the rise in radio-labeled compound concentration. Binding data were analyzed according to the Scatchard plot method and showed the presence of only a single class of high affinity and low capacity binding sites for DHT, with K_d of 8.54 nM and Bmax of 1.81 nM.

The effect of increasing non-radioactive DHT, **1**–**4** concentrations upon [^{3}H]DHT binding to androgen receptor [AR] from prostate was determined. The Ki values for the steroids showed the following order of affinity to AR: **4** > **3** > DHT > **2** > **1** (Figure 3) i.e.

TABLE II Effect of pH on the hamster prostatic 5α-reductase activity

	Testosterone [nM]					
pH	2	20	60	120	Km [nM]	Vmáx (nmol/mg prot/h)
5.0					140	14
% of conversion	2.51	3.26	5.08	2.9		
DHT production (nmol/mg prot/h)	0.2008	2.608	12.192	13.92		
6.0	5.27	1.78	2.08	2.80	22	4.9
% of conversion						
DHT production (nmol/mg prot/h)	0.422	1.424	4.992	13.728		
7.0					5.7	1.2
% of conversion	5.40	0.77	1.33	1.80		
DHT production (nmol/mg prot/h)	0.432	0.616	3.192	4.0		

Results are given in percentage of conversion of T into DHT, and DHT production in nmol/mg of protein/hour

TABLE III IC_{50} value determined for synthesized steroids

Steroid	IC_{50} (μM)
1	280
2	2.6
3	1.6
4	114

IC_{50} value represents the concentration of the steroid that inhibits 50% of 5α-reductase activity and was determined as described in the Experimental section.

The Ki was determined by the following formula:

$$\mathrm{Ki} = \frac{\mathrm{IC_{50}}}{1} + \frac{\mathrm{C}}{\mathrm{Kd}} \quad (1)$$

where IC_{50} = Concentration **1**–**4** required to inhibit the binding of DHT to the androgen receptor by 50%, C = [^{3}H]DHT concentration and Kd = dissociation constant determined from the saturation plot.

DISCUSSION

In this study, we assessed the antiandrogenic activity of four 17α-*p*-substituted benzoyloxy-4-bromo-4-pregnene-3,20-dione derivatives **1**–**4** synthesized by our group,[13] and compared the IC_{50} values obtained for each steroid and its binding affinity for the androgen receptor. These compounds were subcutaneously active, since they decreased the weight of the prostate of gonadectomized hamsters treated with T or DHT (Table I).

Previous studies carried out in our laboratory demonstrated that a bromine atom at the C-6 position of the progesterone skeleton increased the inhibitory power of the progesterone molecule as measured by the conversion of T to DHT.[12] Furthermore, a C-17 benzoyloxy moiety as well as a bromine atom at C-4 in the progesterone skeleton contributed also to the antiandrogenic activity as indicated by the reduction in weight of seminal vesicles, the amount of DHT produced and the decrease in the diameter of the hamster flank organs.[13]

In this study the *in vitro* experiments showed that the novel compounds **1**–**4** are weak inhibitors of 5α-reductase activity. In compound **3** the Br atom in the benzoyloxy side chain and the corresponding chloro compound (**2**) showed much higher 5α-reductase inhibitory activity (lower IC_{50} value) than the corresponding fluoro steroid (**1**) or the methyl substituted benzoyloxy group in **4**.

In this paper, we also evaluated the binding affinity of compound **1**–**4** for the androgen receptor in rat prostate glands. All compounds form a complex with the protein receptor and this explains the fact why the weight of the glands was reduced when the animals were treated with DHT plus steroids **1**–**4**. As a consequence of this, all steroids **1**–**4** function as antagonists for the androgen receptor.

Hamster 5α-reductase enzyme showed a maximum activity at pH 7 with T 2 nM (Figure 2). A comparison of the kinetic parameters at pH of 5 and 7, indicated that the maximum velocity of this enzyme is higher at pH 5 than 7 although affinity for T is higher at pH 7 than 5 (Table II). However the Km and Vmax values (Table II) at pH of 5, 6 and 7 are different. The reason for this discrepancy is unclear

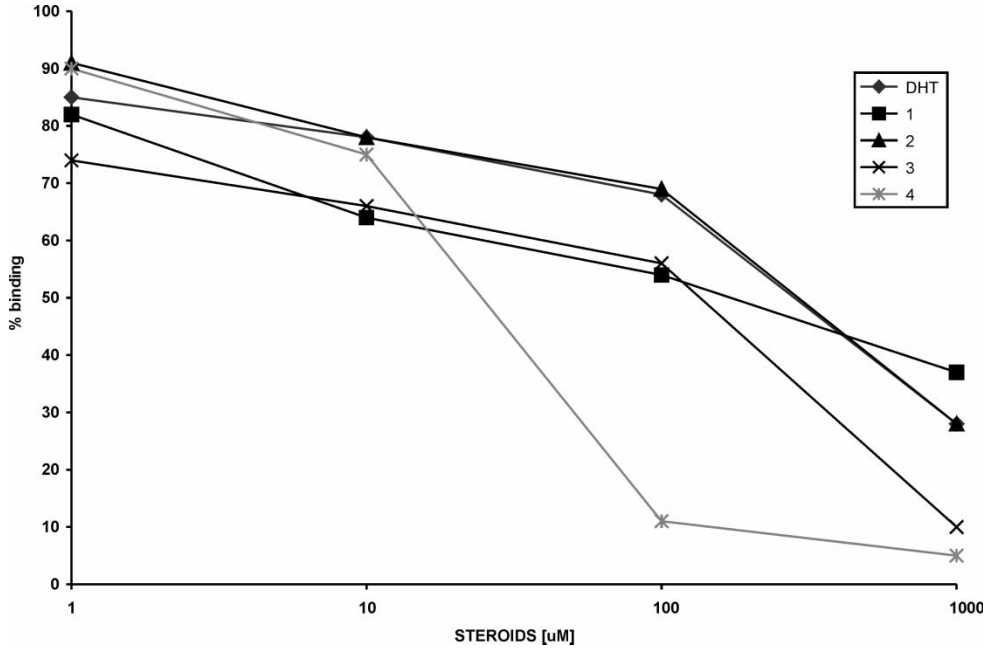

FIGURE 3 Binding specificity. Gonadectomized male hamster seminal vesicles cytosol incubated for 18–20 h at 4°C in presence of 3.15 nM of [^{3}H]DHT and increasing concentrations of unlabeled steroids.

and this phenomenon was also observed by Hirosumi *et al*.[17] and Thigpen *et al*.[21]

It had been reported that the rat prostatic 5α-reductase type 2 enzyme presents maximum activity at a neutral pH, however in this species it has been found that both type 1 and 2 isozymes are present in the prostate and their pH profiles are almost identical to those of the human isozymes.[4,22] Studies with transfected CHO cells have suggested that type 2 isozyme may have a neutral pH optimum in its native state. If these cells are treated with digitonin under conditions in which the plasma membrane is permeabilized but the endoplasmic reticulum membrane left intact, the type 2 isozyme exhibits equivalent activity at both acidic and neutral pH. However physical lysis methods result in a type 2 isozyme with activity only at pH of 5. This isozyme would thus appear to function at a neutral pH within the cell and to shift to a pH 5.0 active form upon cell lysis.[7] The overall data from this study showed the presence of only one prostate isozyme of 5α-reductase, the type 2 whose activity is found between acidic and neutral pHs.

Acknowledgements

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