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RESEARCH ARTICLE

Steroidal 5α-reductase inhibitors using 4-androstenedione as substrate

Marisa Cabeza[1], Karla Valeria Trejo[1], Claudia González[1], Perla García[2], Juan Soriano[3], Yvonne Heuze[1], and Eugene Bratoeff[2]

[1]*Department of Biological Systems and Animal Production, Metropolitan University-Xochimilco, Mexico D. F., Mexico,*
[2]*Department of Pharmacy, Faculty of Chemistry, National University of Mexico City, Mexico D. F., Mexico, and*
[3]*Department of Pathology of the General Hospital of Mexico, Mexico D. F.*

Abstract

The aim of this study was to determine the capacity of some progesterone derivatives, to inhibit the conversion of labeled androstenedione ([^{3}H] 4-dione) to [^{3}H]dihydrotestosterone ([^{3}H]DHT) in prostate nuclear membrane fractions, where the 5α-reductase activity is present. The enzyme 5α-reductase catalyzes the 5α-reduction of 4-dione whereas the 17β-hydroxysteroid dehydrogenase catalyzes the transformation of 4-dione to testosterone or 5α-dione to dihydrotestosterone (DHT). Moreover, we also investigated the role of unlabeled 5α-dione in these pathways. In order to determine the inhibitory effect of different concentrations of the progesterone derivatives in the conversion of [^{3}H] 4-dione to [^{3}H]DHT, homogenates of human prostate were incubated with [^{3}H] 4-dione, NADPH and increasing concentrations of non-labeled 5α-dione. The incubating mixture was extracted and purified using thin layer chromatography. The fraction of the chromatogram corresponding to the standard of DHT was separated and the radioactivity determined. The results showed that the presence of [^{3}H] 4-dione plus unlabelled 5α-dione produced similar levels of DHT as compared to [^{3}H] 4-dione. On the other hand, the results indicated that 17α-hydroxypregn-4-ene-3,20-dione **5** and 4-bromo-17α-hydroxypregn-4-ene-3,20-dione **7b**, were the most potent steroids to inhibit the conversion of [^{3}H] 4-dione to [^{3}H]DHT, showing IC_{50} values of 2 and 1.6 nM, respectively.

Keywords: Androstenedione pathway, 5α-reductase, prostate, DHT conversion from 5α-dione, 5α-reductase inhibitors, testosterone pathway

Introduction

Testosterone (T) plays an important role in the testes and muscle; on the other hand dihydrotestosterone (DHT) is crucial for the development, function and pathology of the prostate. This tissue produces DHT from the reduction of the circulating T and such conversion is carried out by the 5α-reductase enzyme present in the prostate. T or DHT activate the androgen receptor (AR) which interacts with androgen response elements in DNA to regulate gene transcription[1].

In addition to T, the adrenal androgen androstenedione (4-dione) is converted to 5α-androstanedione (5α-dione) by the steroid 5α-reductase present in the prostate[2]. After castration the steroid 4-dione has been implicated as a source of DHT in prostate tissue[3]. The presence of other steroidogenic enzymes in the prostate[4], as well as the availability of various steroid precursors such as 4-dione suggest the existence of additional pathways involved in the biosynthesis and metabolism of DHT (Figure 1).

The activity of 5α-reductase enzyme (EC 1.3.99.5) in androgen dependent tissues has long been known. Two isoforms of 5α-reductase had been identified: named 1 and 2, each encoded by different genes, which have been characterized in several species[5,6]. 5α-Reductase type 2 isozyme plays a major role in prostate cancer and benign prostatic hyperplasia as it is predominantly expressed in this tissue. It is well known that type 1 is expressed in prostate epithelial cells while the type 2 is mainly located

Address for Correspondence: Marisa Cabeza, Department of Biological Systems and Animal Production, Metropolitan University-Xochimilco, Calzada del Hueso no. 1100, México, D.F. C.P 04960, México. Phone: (011-52-55) 5483-72-60, Fax: (52-55) 5483-72-60. E-mail: marisa@correo.xoc.uam.mx

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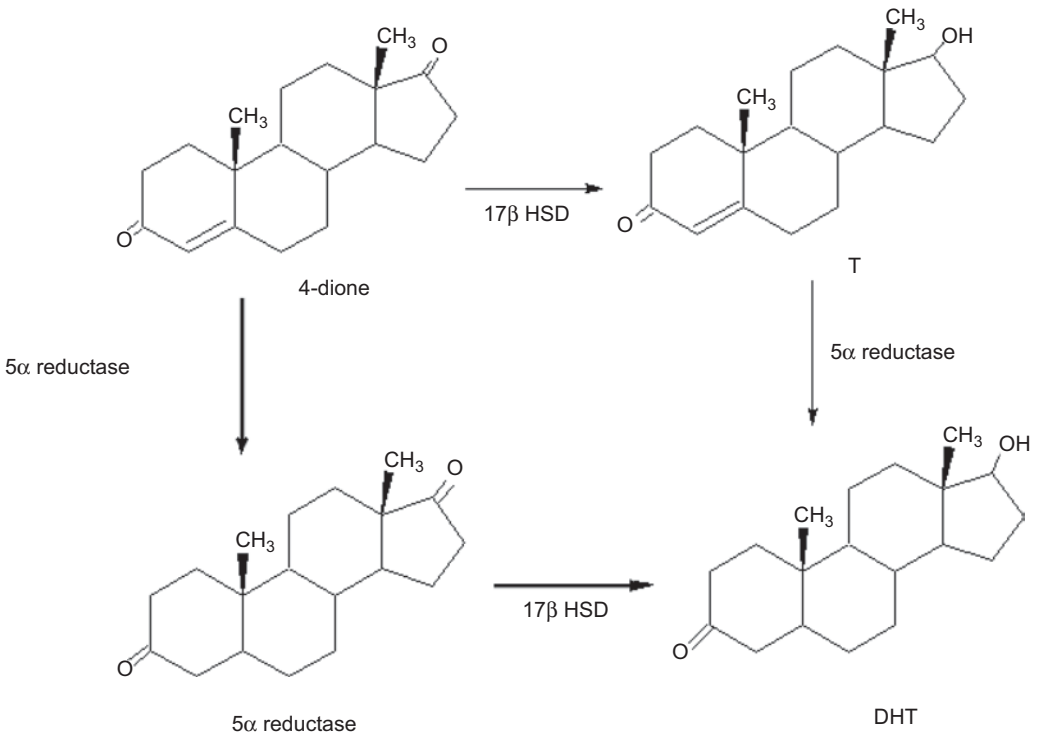

Figure 1. Metabolic pathways for the biosynthesis of DHT in the prostate. The steps that do not require the presence of T are indicated in bold lines.

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in the stromal compartment[7,8]. 5α-Reductase type 1 is also present in the liver, skin and acts in a neutral or basic medium, whereas type 2 is active in acidic pH[5]. Recently, a type 3 5α-reductase had been described in prostate cancer cells[9] and in a human sebaceous gland cell line[10].

In this study, we determined the biosynthesis of [^{3}H]DHT from [^{3}H] T or [^{3}H] 4-dione in the presence or absence of non-labeled DHT or 5α-dione in the incubation medium. These studies were carried out using human 5α-reductase from nuclear membrane fractions of prostate. The enzyme 5α-reductase catalyzes the 5α-reduction of 4-dione whereas the 17β-hydroxysteroid dehydrogenase catalyzes the transformation of 4-dione to T or 5α-dione into DHT. Moreover, we assessed the effect of different inhibitors of T 5α-reductase as finasteride and the progesterone derivatives: 3, 20-dioxopregn-4-ene-17-yl acetate **4,** 17α-hydroxypregn-4-ene-3,20-dione **5**, 17α-hydroxy-4,5-epoxypregnane-3,20-dione **6**, 4-chloro-17α-hydroxypregn-4-ene-3,20-dione **7a**, 4-bromo-17α-hydroxypregn-4-ene-3,20-dione **7b**, 4-chloro-3,20-dioxopregn-4-ene-17-yl-4-ethylbenzoate **8a** and 4-bromo-3,20-dioxopregn-4-ene-17-yl-4-ethylbenzoate **8b** as 4-dione 5α-reductase inhibitors. These compounds were synthesized and evaluated in our laboratory as inhibitors of both isozymes of T 5α-reductase[11].

Materials and methods

Materials

(1, 2, 6, 7-^{3}H) Testosterone [^{3}H] T specific activity: 95 Ci/mmol and androstenedione (androst-4-ene-3, 17-dione [1,2,3,7-^{3}H(N)] [^{3}H] 4-dione (Figure 1) specific activity 90 Ci/mmol were provided by Perkin Elmer Life and Analytical Sciences. (Boston, MA). Radioinert T, 5α-DHT, androstenedione and androstanedione were supplied by Steraloids (Wilton, NH). Sigma Chemical Co. (ST. Louis, Mo) provided NADPH. Finasteride was obtained by extraction from Proscar (Merck, Sharp and Dohme). The tablets were crushed, extracted with chloroform and the solvent was eliminated in vacuum; the crude product was purified by silica gel column chromatography. The melting point of the isolated finasteride (252–254°C) was identical to that reported in the literature.

Steroids **4–6**, **7a**, **7b**, **8a** and **8b** (Table 3) were synthesized according to the method of Bratoeff et al.[11]

Method

The prostate of a man 37-years old, who died from gastrinoma was extirpated and the tissue was immediately chilled in ice-cold 150 mM NaCl and stored at –20°C. Frozen human prostate was thawed on ice and minced with a tissue mill IKA A11 basic. Unless specified, the following procedures were carried out at 4°C.

Human prostate tissue was homogenized in 2 volumes of buffer A (20 mM sodium phosphate, pH 6.5 containing 0.32 M sucrose, 0.1 mM dithiothreitol Sigma-Aldrich Inc.) with a tissue homogenizer Ultra-Turrax IKA, T18 basic. (Wilmington, NC). Homogenates were centrifuged at 1500*g* for 60 min at 0°C[12,13] in a SW 60 Ti rotor (Beckman instruments, Palo Alto, CA). The pellets were separated, suspended in medium A and kept at –70°C.

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The suspension, 5 mg of protein/mL for human prostates, determined by the Bradford method[14] was used as source of 5α-reductase.

Determination of 5α-reductase activity

The activity of the T 5α-reductase or 4-dione 5α-reductase was assayed as previously described[12,13]. The reaction mixtures contained a final volume of 1 mL, 1 mM DTT, sodium phosphate buffer 40 mM, at pH 6.5, 2 mM, NADPH, 2 nM [1,2,6,7-^{3}H]T or [1,2,3,7-^{3}H(N)] 4-dione and the prostatic enzyme fractions. The amount of prostatic enzyme fractions was determined to adjust the rate of conversion of T to DHT or 4-dione to 5α-dione to around 28%. T, DHT, unlabeled 4-dione or 5α-dione concentrations were adjusted to 25–250 nM in the incubating medium, by adding one of the following reagents cold T, DHT, unlabeled 4-dione or 5α-dione. The reactions in duplicate were started when added to the enzymatic fraction (400 μg protein in a volume of 80 μL) incubated at 37°C for 60 min[13] and stopped by mixing with 1 mL of dichloromethane. Incubation without tissue was used as a control. All reactions were carried out in two different times by duplicate.

Extraction and purification of steroids formed from T and 4-dione

The mixtures (incubation medium/dichloromethane), were agitated on a vortex for 1 min and the dichloromethane phase was separated and placed in another tube. This procedure was repeated four more times. The dichloromethane extract was evaporated to dryness under a nitrogen stream and suspended in 50 μL of methanol that was spotted on HPTLC Keiselgel 60 F_{254} plates. T, DHT, 4-dione and 5α-dione were used as standard carriers and were applied in different lanes on both lateral sides of the plates (T, T+DHT, DHT and 4-dione, 4-dione+5α-dione, 5α-dione). The plates were developed in chloroform-acetone 9:1 and were air-dried; this procedure was repeated two more times. The steroidal standard DHT carrier was detected using phosphomolibdic acid reagent DHT; T and 4-dione with an UV lamp (254 nm). The 5α-dione standard was detected by the Zimmermann reaction; the lines of the plate corresponding to 5α-dione were sprayed with a freshly prepared mixture of equal volume of a 2% solution of m-dinitrobenzene in absolute ethanol and a 2.5 N solution of potassium hydroxide. After the plates were segmented in areas of one cm each, they were cut off and the strips soaked in 5 mL of Ultima Gold (Packard). The radioactivity was determined in a scintillation counter (Packard tri-carb 2100 TR). in the segment corresponding to T, DHT, 4-dione and 5α-dione carriers. The radioactivity that had identical chromatographic behavior as the T, DHT and 5α-dione standards was considered as the T, DHT or 5α-dione transformation. Control incubations, chromatography separations and identifications, were carried out in the same manner as described above except that the tubes did not contain tissue. The T, DHT or 5α-dione transformation yields were calculated from the strips, taken into account the entire radioactivity on the plate.

The T 5α-reductase or 4-dione 5α-reductase activity was calculated from the percentage of labeled T, DHT, or 5α-dione formed, taking into consideration recovery, blank values, the specific activity of [^{3}H] T or [^{3}H] 4-dione and the radio of added [^{3}H] T or [^{3}H] 4-dione to unlabeled T, 4-dione, DHT or 5α-dione. Km and V_{max} values were derived from Linewear–Burk plots and determined also by the Michaelis–Menten expression[15,16].

The efficiency of T 5α-reductase and 4-dione 5α-reductase were estimated according to Weisser and Krieg report[2].

Determination of 50% of the inhibitory concentration of finasteride and steroids **4, 5, 6, 7a, 7b, 8a** and **8b** in 5α-reductase activities

In order to calculate the IC_{50} values (the concentration of finasteride and steroids **4, 5, 6, 7a, 7b, 8a** and **8b**, Table 3) required to inhibit 50% of the activity 5α-reductase, six series of tubes containing increasing concentrations of these steroids (10^{-11} – 10^{-3} M) were incubated in duplicate, in the presence of: 1 mM of dithiothreitol, 40 mM sodium phosphate buffer pH of 6.5; 2 mM NADPH, 2 nM [^{3}H] 4-dione plus 125 nM of 5α-dione and 400 μg of protein from the enzymatic fraction obtained from human prostate as described above.

The reactions were carried out in duplicate at 37°C for 60 min and 1 mL of dichloromethane was added to stop the reaction. The extraction and the chromatographic procedures were carried out as described above. The radioactivity contained in the fraction corresponding to DHT carrier was determined using the above procedure.

Results

In vitro experiments

5α-reductase activity

Human prostatic 5α-reductase from nuclear membrane fractions of prostate affects the conversion of T to DHT in the presence of NADPH (Table 1). Furthermore, labeled T in the presence of increasing concentrations of unlabeled DHT showed higher conversion to labeled DHT (higher 5α-reductase activity) V_{max} =103.9 pmol/mg of protein/h as compared to the experiment with increasing concentrations of T (V_{max} = 59.6 pmol/mg of protein/h). On the other hand, the 5α-reductase enzyme showed a higher affinity (minor Km value, Table 1) when DHT was present in the incubating medium as compared to the presence of T (major Km value, Table 1).

It was observed that after the incubation with labeled 4-dione, a radioactive zone that had identical chromatographic behavior as the standard of 5α-dione (Rf value of 0.87), was found, thus demonstrating the activity of 5α-reductase (Table 1). When increasing concentrations of unlabeled 5α-dione were added to the incubation medium, a higher production of labeled 5α-dione (67.5 pmol/mg of protein/h) was observed (Table 1).

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However, when increasing concentrations of labeled and unlabeled 4-dione were incubated, a lower rate of 5α-dione formation was observed (26.10 pmol/mg of protein/h), Table 1. In addition, the Km values for 5α-reductase in the presence of unlabeled 5α-dione were lower as compared to the Km values in the absence of this steroid (Table 1). On the other hand, 4-dione possesses a lower affinity than T for 5α-reductase as shown by their Km values of 176 nM for 4-dione and 96.29 nM for T (Table 1).

When prostatic nuclear membrane fraction was incubated in the presence of labeled 4-dione plus increasing concentrations of unlabeled 5α-dione, a slightly higher conversion to labeled DHT was found. The V_{max} of conversion to DHT was in the range of 19.2 pmol/mg of protein/h, when increasing concentrations of 4-dione was incubated alone a similar conversion to DHT was found; the V_{max} determined for DHT was of 18 pmol/mg of protein/h. (Table 2). Therefore it appears that the 5α-dione is converted to DHT by a catalytic reaction mediated by 17β-HSD[4].

In addition to the labeled DHT formed from labeled 4-dione, we also found a radioactive zone that had identical chromatographic behavior as the carrier T (Rf value of 0.59); these results suggest that 17β-HSD (Table 2) is present in the prostate tissue as had been previously reported[17]. However the V_{max} value (9.7 pmol/mg of protein/h) for T produced from labeled 4-dione was higher than the V_{max} value (1.9 pmol/mg of protein/h) for T obtained, when unlabeled 5α-dione was present in the incubated medium (Table 2). These results imply that 5α-dione could have inhibited the 17β-HSD activity. However the presence of unlabeled 5α-dione increased the affinity of 17β-HSD for its substrate (minor Km value) as compared to the Km value obtained when T was produced from 4-dione alone (see Table 2). It is remarkable

Table 1. DHT and 5α-dione production from different concentrations of labeled T and 4-dione by human prostate homogenates. Effect of increasing concentrations of unlabeled DHT and 5α-dione on the prostatic 5α-reductase activity.

Substrate	Concentration of the substrate (nM)					V_{max}	Km nM (pmol/mg of protein/h)
1. Labeled testosterone	2	55	108	148	203		
Production of labeled DHT (pmol/mg of protein/h)	0.35 ± 0.06	21.7 ± 7.2	48.9 ± 14.1	88.2 ± 14.1	122 ± 19.5	**96.29**	**59.6**
2. 2 nM of labeled T plus increasing concentrations of DHT							
Production of labeled DHT (pmol/mg of protein/h)	0.57 ± 0.23	39 ± 12.32	90.3 ± 30.7	153.3 ± 57.7	194.6 ± 70.79	**89.2**	**103.9**
3. Labeled 4-dione							
Production of labeled 5α-dione (pmol/mg of protein/h)	0.075 ± 0.02	5.96 ± 0.20	9.9 ± 1.3	30.6 ± 9.2	40.3 ± 6.2	**176**	**26.10**
4. 2 nM of labeled 4-dione plus increasing concentrations of 5α-dione							
Production of labeled 5α-dione (pmol/mg of protein/h)	0.76 ± 0.06	21.4 ± 4.2	45.2 ± 9.1	48.1 ± 10.1	52.1 ± 2.3	**58.9**	**67.5**

Table 2. T and DHT production from different concentrations of labeled 4-dione by human prostate homogenates. Effect of increasing concentrations of unlabeled 5α-dione.

SUBSTRATE	Concentration of the substrate (nM)					V_{max}	Km nM (pmol/mg of protein/h)
1. Labeled 4- dione	2	55	108	148	203		
Production of labeled T (pmol/mg of protein/h)	0.07 ± 0.01	1.01 ± 0.025	2.35 ± 0.01	4.03 ± 0.35	4.03 ± 0.2	**331**	**9.7**
Production of labeled DHT (pmol/mg of protein/h)	0.095 ± 0.06	7.025 ± 0.95	14.14 ± 10.4	25.77 ± 16.35	28.0 ± 11.79	**86.45**	**18**
2. 2 nM of labeled 4-dione plus increasing concentrations of 5α-dione							
Production of labeled T (pmol/mg of protein/h)	0.01 ± 0.001	0.67 ± 0.02	0.69 ± 0.06	2.01 ± 0.4	2.1	**188.3**	**1.9**
Production of labeled DHT (pmol/mg of protein/h)	0.067 ± 0.08	8.6 ± 0.02	15.2 ± 5.6	22.8 ± 0.09	33.6 ± 2.5	**69**	**19.2**

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to indicate that 17β-HSD is a labile enzyme, whose activity can easily be lost in the homogenizing process[18]; this fact could explain the low rate of conversion of 4-dione to T.

The 5α-reductase's enzymatic efficiency (V_{max}/Km)[16,19] obtained from the rate between maximal velocities (V_{max}) of production of DHT or 5α-dione, using different substrates and the Michaelis constants (Km) are shown in Figure 2. V_{max}/Km had up to 1.9 fold higher efficiency when [^{3}H]T plus DHT were used as substrate as compared to T alone. Furthermore V_{max}/Km had up to approximately 9 fold higher efficiency when [^{3}H]4-dione plus 5α-dione were used as substrate as compared to 4-dione. The experimental results in this study indicated that human prostate 5α-reductase converts in a similar manner 4-dione to 5α-dione as compared with T to DHT under these experimental conditions (Figure 2).

IC_{50} values of steroidal compounds in human prostate

The concentrations of finasteride and the progesterone derivatives **4**, **5**, **6**, **7a**, **7b**, **8a** and **8b** required for inhibiting 4 dione 5α-reductase activity by 50% (IC_{50}) were determined from the inhibition plots using different concentrations of the tested steroids (Figure 3); these results are shown in Table 3. The data in this table show very clearly the inhibitory effect of finasteride as well as steroids **4**, **5**, **7a**, **7b**, **8a** and **8b** on human prostate 5α-reductase when labeled 4-dione plus 125 nM of unlabeled 5α-dione were used as substrate. However compound 6 did not showed any inhibition of the enzyme activity.

Discussion

In this article, we demonstrated that T and 4-dione 5α-reductase activity, obtained from nuclear membrane fractions of prostate was increased in the presence of its own products of reaction, such as DHT or 5α-dione. Furthermore, the efficiency of these reactions was higher in the presence of their products; this evidence indicates that 5α-reductase is an allosteric enzyme[16] activated by its own products of reaction. This fact could have important implications since overabundance of DHT has been implicated in the pathogenesis of benign prostatic hyperplasia and prostate cancer[20]. 5α-Reductase has a biologically important role since it is responsible for concentrate intraprostatic DHT when/if serum T levels are physiologically low[21]. Experimental studies had suggested that T is more potent than DHT in stimulating the expression of many androgen-response genes in regressed prostate[22]. The expression of these androgen-response genes is likely to be associated with androgen-dependent prostate preservation, since these genes preserve the capacity of the prostatic cells to undergo apoptosis and are less associated with a stimulating proliferation during prostate growth. On the other hand, high levels of intracellular DHT result in cellular proliferation and delay in cellular differentiation[22].

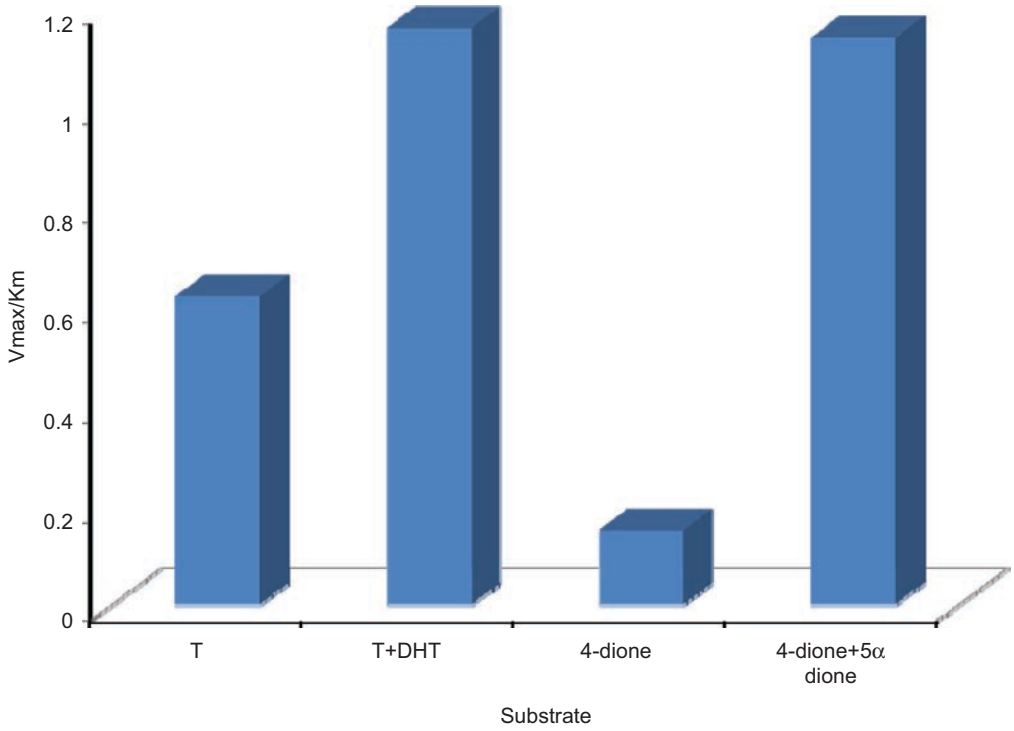

Figure 2. Enzymatic efficiency (V_{max}/Km) was obtained from the rate between maximal velocities (V_{max}) of DHT or 5α-dione produced, using different substrates and the Michaelis constants (Km). V_{max}/Km was higher when [^{3}H]T+DHT were used as substrate than when T was used as a substrate. Furthermore, V_{max}/Km was higher also when [^{3}H]4-dione+5α-dione were used as substrate than when 4-dione was used. However, human prostate 5α-reductase enzyme converts 4-dione to 5α-dione in a similar manner as compared to T to DHT.

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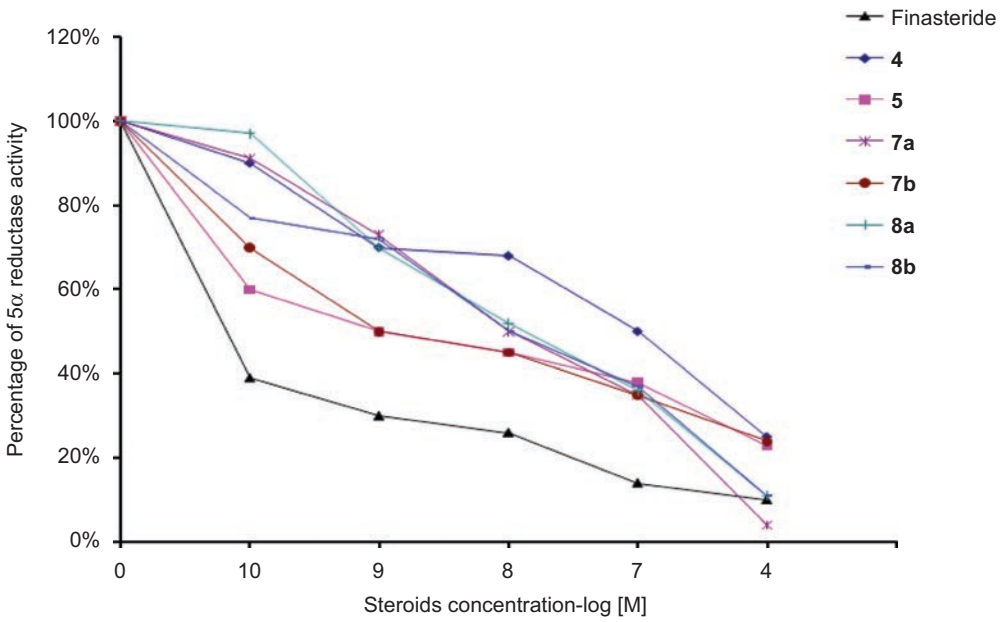

Figure 3. Inhibition plots using different concentrations of the tested steroids against the percentage of activity of 4-dione 5α-reductase. These plots were used for the determination of the concentrations of finasteride as well as the progesterone derivatives **4**, **5**, **6**, **7a**, **7b**, **8a** and **8b** required for inhibiting 4-dione 5α-reductase activity by 50%.

Some evidences indicated that 5α-reductase prefers 4-dione as a substrate than T[23,24]. This is evidenced by the low free levels of T in the circulation compared to 4-dione and also by higher affinity of T to AR than for the 5α-reductases. These data suggest that the 5α-reductase activity step precedes the 17β-HSD activity[4,18] according to the pathway 4-dione →5α-dione →DHT. On the basis of this phenomenon, it is important to consider this source of DHT in benign prostatic hyperplasia and prostate cancer. In this sense the results presented in this article indicated that human prostate 5α-reductase converts T to DHT more efficiently than 4-dione to 5α-dione; these data are in agreement with those previously reported by Weisser and Krieg[2]; however in contrast to our data, the studies of Andersson and Russell[22] using cloned and expressed human steroid 5α-reductases indicated that human 5α-reductase catalyzes more efficiently the conversion of 4-dione than that for T. These differences in the results could be explained by considering the differences in the experimental protocols, such as: pH, the use of the nuclear membrane fractions from human prostate instead off transfected cells. However, the results obtained in this study showed that the 4-dione possesses a lower affinity than T for 5α-reductase as indicated by their Km values.

It is important to observe that in this experiment the V_{max} of 4-dione conversion to T was low (9.7 pmol/mgof protein/h), probably due to the labile nature of 17βHSD[18]. Furthermore, V_{max} was still lower when 5α-dione was present in the incubating medium (1.9 pmol/mg of protein/h); this result can be explained on the grounds that 5α-dione could inhibit the activity of the 17βHSD enzyme. However, in this experiment, the amount of T obtained from 4-dione was so low under our experimental conditions that it contributed very little to the pool of DHT quantified in this experiment.

Previously, it had been reported that in benign prostatic hyperplasia's stroma cells produce more 5α-reduced metabolites (DHT and 5α-dione) than normal prostatic's stroma cells[2]. On this basis and taking into account our results, it could be assumed that a positive feedback of 5α-reductase activity could be occurring in this pathology, since a higher amount of reduced metabolites produced enhanced 5α-reductase activity. As a result of this, increased concentrations of DHT could produce a cellular proliferation and delay in cellular differentiation[22].

The results obtained in these experiments showed also, that 4-dione 5α-reductase from normal prostate was inhibited by finasteride and steroids **4**, **5**, **7a**, **7b**, **8a** and **8b**, which have demonstrated its activity as inhibitors of T 5α-reductase types 1 and 2[11]. On the other hand the inhibitory effect of finasteride on 5α-reduction of T and 4-dione had previously been reported in benign prostatic hyperplasia, BPH[25–27]. Finasteride is a better inhibitor of T 5α-reductase type 2 than type 1[5]; in this experiment we found a lower IC_{50} value for finasteride as compared to 4-dione when it was used as substrate. When T was used as substrate, under the same experimental conditions (type 2 enzyme) a higher IC_{50} value was obtained[11].

In the experimental conditions steroids, 17α-hydroxypregn-4-ene-3,20-dione **5** and the 4-bromo-17α-hydroxypregn-4-ene-3,20-dione 7b were the most potent steroids for the inhibition of the transformation of [^{3}H]

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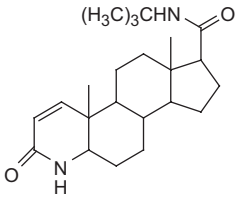
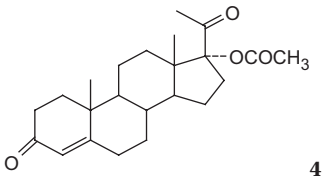
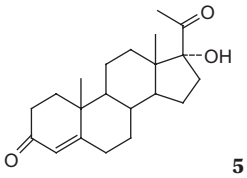
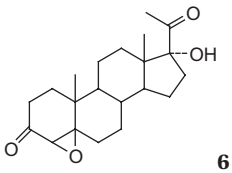
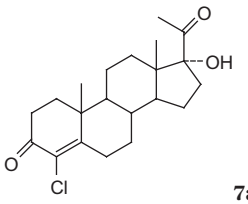
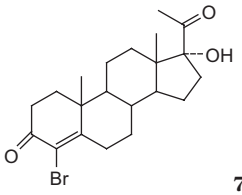
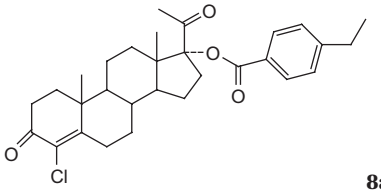
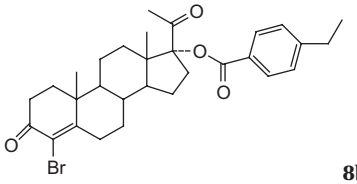
Table 3. Effect of different compounds as inhibitors of the activity of 5α-reductase.

Steroid structure	IC_{50} (nM)
 **Finasteride**	1.3 ± 0.4
 **4**	500 ± 30
 **5**	2 ± 0.6
 **6**	NA
 **7a**	63 ± 1.2
 **7b**	1.6 ± 0.4
 **8a**	73 ± 9
 **8b**	50 ± 6

5α-reductase was obtained from the pellet fraction of homogenates of human prostate centrifuged at 1500*g*. The homogenates were incubated with labeled 4-dione plus 125 nM of unlabeled 5α-dione and increasing concentration of the synthesized compounds. The results show the concentration of the steroids necessary for inhibiting 50% of the activity of 4-dione 5α-reductase (IC_{50}). NA, non-active compound.

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4-dione to [^{3}H]DHT, showing IC_{50} values of 2 and 1.6 nM, respectively.

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Declaration of interest

We report in this manuscript that we don't have any conflict of interest with this research work.

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