5α-Reductase Inhibitory and Antiandrogenic Activities of Novel Steroids in Hamster Seminal Vesicles

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The pharmacological activity of several 16-bromosubstituted trienediones 4 and 5, 16-methyl substituted dienediones 6 and 7 and the 16-methyl substituted trienedione 8 was determined on gonadectomized hamster seminal vesicles by measuring the in vitro conversion of testosterone (T) to dihydrotestosterone (DHT) as 5α-reductase inhibitors and also the ability of these steroids to bind to the androgen receptor. Steroids 6 and 7 when injected together with T decreased the weight of the seminal vesicles thus showing an antiandrogenic effect. Compounds 5 and 6 reduced substantially the conversion of T to DHT and therefore can be considered good inhibitors for the enzyme 5α-reductase; however both steroids failed to form a complex with the androgen receptor. On the other hand compound 7 showed a very small inhibitory activity for the enzyme 5α-reductase, exhibited a very high affinity for the androgen receptor and thus can be considered an effective antiandrogen. This compound also reduced substantially the weight of the seminal vesicles. Steroids 4 and 8 did not reduce the weight of the seminal vesicles and exhibited a low affinity for the androgen receptor; 8 showed a weak 5α-reductase inhibitory activity, whereas 4 exhibited a weak antiandrogenic effect.

Key words: seminal vesicle; 5α-reduction; 5α-reductase; androgen receptor; T to DHT conversion; C-16 substituent

Steroid 5α-reductase (EC 1-3-99-5) is a NADPH dependent enzyme which converts testosterone (T) into dihydrotestosterone (DHT) 2, 3. This enzyme is located in the androgen-dependent tissue such as prostate, seminal vesicles, epididymis and the skin. It is believed that DHT plays a role in pathological conditions such as benign prostatic hyperplasia (BHP), prostatic cancer, acne, female hirsutism and male pattern baldness.4, 5

The essential role of 5α-reductase in prostate growth has been determined by the discovery of 5α-reductase deficiency in male pseudohermaphrodism patients who had atrophic prostate glands despite normal testosterone level.3, 4 DHT is the most active agonist for the androgen receptor5 although T also mediates the androgenic action in the tissues. Various steroidal compounds have been developed as 5α-reductase inhibitors for the medical therapy of BHP.5 Finasteride 3 (Fig. 1) a 4-aza steroid is the most extensively studied class of 5α-reductase inhibitors.7 This compound is the first approved 5α-reductase inhibitor in the U.S.A. for the treatment of BHP. This drug has approximately a 100-fold greater affinity for type 2—5α-reductase than for type 1. In humans finasteride decreases prostatic DHT level and reduces prostate size, while T tissue concentration remains constant.5

In this study, we determined the antiandrogenic activity of five new steroidal compounds: 4—8 in gonadectomized hamster seminal vesicles.

Seminal vesicles are male accessory glands that are androgen-dependent. These organs are capable of reducing T to DHT in both intact and gonadectomized animals and have also been used for evaluation of steroidal and non steroidal compounds as antiandrogens.8, 9

Chemistry Several years ago we synthesized several new 17α-acryloyloxy-16β-methyl substituted pregnadiene-3,20-dione derivatives.10 Some of these compounds showed a high inhibitory effect for the enzyme 5α-reductase as well as high affinity for the androgen receptor. On the basis of these results and in order to expand the scope of this study, recently we synthesized several new 17α-acryloyloxy-16β-substituted (methyl, bromine) pregnadiene-3,20-diones and pregnatriene-3,20-triones (4—8) which are described in this paper.

Synthesis of Steroids 4—8 Compounds 4—8 (Fig. 1) were prepared from the commercially available 16-dehydrogenolone acetate (Fig. 2). Epoxidation of the double bond at C-16 with hydrogen peroxide and sodium hydroxide afforded the epoxy derivative 9. The bromohydrin 10 was obtained when the intermediate 9 was treated with hydrobromic acid (32%) in dioxane for 6 h. When compound 10 was allowed to reflux with dichloro-dicyanobenzoquinone (DDQ) in dioxane for 4 d, the trienedione 4 was obtained. Esterification of the hydroxyl group in 4 with trifluoroacetic anhydride, p-toluenesulfonic acid and cyclopentanecarboxylic acid yielded the desired ester 5.

Compounds 6—8 (Fig. 1) were prepared from the epoxy derivative 9 (Fig. 3). Acetylation of 9 in the usual manner yielded the acetoxy compound 11. The protection of the carbonyl group in 11 was effected with ethylene glycol, trimethyl orthofomate and p-toluenesulfonic acid (PTSA). The resulting dioxolane derivative 12 was allowed to reflux with methylmagnesium chloride in tetrahydrofuran (THF); this reaction afforded the 16-methyl substituted dioxolane derivative 13. Hydrolysis of the dioxolane ring in 13 to recover the carbonyl moiety was carried out with PTSA in acetone thus forming the carbonyl derivative 14. The oxidation of the hydroxyl group at C-3 in 14 was effected with lithium carbonate, lithium bromide and bromine in N,N-dimethylforformamide (DMF). This reaction afforded the 4,6-diene-3,20-dione moiety 15. Esterification of the hydroxyl group in 15 with trifluoroacetic anhydride, cyclopentylacetic acid and PTSA yielded the desired ester 6. When alcohol 15 was treated in the same manner with cyclopentane carboxylic acid, the corresponding cyclopentanecarboxylate ester 7 was...
Fig. 1. Steroid Structures

Fig. 2. Synthesis of New Steroids

Fig. 3. Synthesis of New Steroids
obtained.

Treatment of 6 with 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ) in dioxane afforded the trienedione compound 8.

**Biological Activity** The biological activity of steroids 4 to 8 were determined in gonadectomized male hamsters divided in several groups. The animals in the control group were injected with the vehicle (sesame oil); in the other groups they were treated with T, a combination of T with finasteride or T with the new steroids. After 6 d of treatment, the animals were anesthetized with ether and sacrificed. The seminal vesicles were removed weighed, homogenized and used for the *in vitro* experiments.

**Seminal Vesicles** After castration the weight of the seminal vesicles of the male hamsters significantly decreased (*p*<0.05) as compared to that of the normal glands. Treatment with vehicle alone (control) did not change this condition whereas subcutaneous injections of 200 μg of 1 for 6 d significantly increased (*p*<0.05) the weight of the seminal vesicles in castrated male hamsters (141.7 mg) (Table 1). When 1 and finasteride 3 were injected together, the weight of the seminal vesicles decreased (120 mg). The injection of steroids 6 and 7 together with T decreased the weight of the seminal vesicles as compared to the T treated hamsters thus suggesting an inhibitory effect on 5α-reductase enzyme type 2. The most effective compounds showing the highest inhibitory effect for 5α-reductase enzyme were steroids 6 and 7 having the lowest value of the weight of the seminal vesicles, 118 and 115 mg respectively.

**In Vitro Metabolism of T to DHT** Since the weight of the seminal vesicles depends on the 5α-reduced androgens, it was important to determine the effect of the new steroids 4—8 on the conversion of 1 to 2 *in vitro* metabolism of [3H]T to [3H]DHT in seminal vesicles homogenates of castrated male hamsters at pH of 6 in the presence of NADPH. The extracts from castrated male hamster seminal vesicles were subjected to TLC analysis. The zone corresponding to DHT (2) standard (Rf value of 0.33) of each experimental chromatogram was eluted and the radioactivity determined. The results (Fig. 4) obtained from two separate experiments performed in duplicate demonstrated a lower [3H]T to [3H]DHT conversion in the T with finasteride treated animals than testosterone alone thus indicating that finasteride 3 is a good inhibitor for the conversion of T to DHT at pH of 6. Figure 4 also shows the effect of the new steroids 4—8 on the rate of DHT formation expressed as fmoles of DHT/mg of protein/h. The four compounds (5—8) produced a much lower concentration of DHT as compared to testosterone. On the other hand, compounds 5, 6 and 8 exhibited a lower DHT conversion than finasteride 3 thus showing a high 5α-reductase inhibitory effect. Steroid 4 gave a higher *in vitro* conversion of 1 to 2 (*p*<0.05) than the other steroids thus showing a weak androgenic effect.

Compound 7 which is structurally very similar to steroid 6 (one carbon atom less) showed a higher *in vitro* conversion of 1 to 2 (*p*<0.05) than steroids 3, 5, 6 and 8 however it still can be considered a 5α-reductase inhibitor due to the lower production of DHT as compared to testosterone.

**Scatchard Analysis of Receptor Androgen Binding** When cytosol aliquots (2.64 mg protein) obtained from gonadectomized male hamster seminal vesicles homogenates were incubated with increasing concentrations of [3H]DHT, a typical saturation curve was obtained. The levels of the non-specific bound hormone increase progressively with the rise of radiolabeled compound concentration. Data were analyzed according to the Scatchard plot method. A linear relationship indicates the presence of only one single class of high affinity and low capacity of binding sites with *Kd* and *Bmax* values of 1.26 and 0.615 nm, respectively, for this tissue (Fig. 5).

**Competition Analysis of Synthesized Steroids for Androgen Receptors (ARs)** The effect of increasing concentrations of nonradioactive synthetic steroids upon [3H]DHT binding to androgen receptors from male hamster seminal vesicles in two different experiments are presented in Fig. 6. The *K* values for the synthesized steroids showed the follow-

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<td><strong>Treatment</strong></td>
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The weight of seminal vesicles were measured from animals that received sc treatment of C-16 substituted steroids.
ing order of affinity to ARs: DHT > 7 > 4 > 8 with values of 8.29, 11.79, 45.38 and 73.04 respectively. Compounds 5 and 6 did not show any affinity for the androgen receptors.

**Discussion**

This study reports the 5α-reductase inhibitory activity and the antiandrogenic effect of novel 16-bromo substituted trienediones 4, 5, the 16β methyl substituted dienediones 6, 7 and the trienedione 8 (Fig. 1). Compound 4 (Fig. 4) did not decrease the weight of the seminal vesicles and did not inhibit the conversion of T to DHT (it has a weak androgenic effect) however it competed for the ARs (Ki, 45.38 nm). On the other hand, steroids 5, 6 and 8 showed a 5α-reductase inhibitory effect slightly higher than the commercially available finasteride 3 presently used for the treatment of benign prostatic hyperplasia however compounds 5 and 8 did not decrease the weight of the seminal vesicles compounds 5 and 6 did not show any affinity for the androgen receptor as indicated previously, however they strongly inhibited the enzyme 5α-reductase. Steroid 8 showed a slight affinity for the ARs, a moderate 5α-reductase inhibitory activity and did not decrease the weight of the seminal vesicles. Compound 7 which has a low inhibitory activity for the conversion of T to DHT and is structurally similar to 6 binds with a high affinity to the ARs and reduces the weight of the seminal vesicles, thus showing high antiandrogenic and also an inhibitory effect for the enzyme 5α-reductase.

In addition, we demonstrated that radio-inert DHT in increasing concentrations, inhibited the binding of [3H]DHT to the ARs present in cytosolic fraction as shown by the Ki value of 8.29 nm (Fig. 6). Furthermore, some radio-inert synthetic steroids (4, 8, 7) also inhibited the binding of [3H]DHT to the cytosolic ARs, as indicated by the respective Ki values. In contrast steroids 5 and 6 did not have any inhibitory effect on cytosolic ARs, as shown in Fig. 6.

**Experimental**

**Chemical and Radioactive Material**

Solvents were laboratory grade or better. Melting points were determined on a Fisher Johns melting point apparatus and are uncorrected. 1H- and 13C-NMR were taken on Varian gemini 200 and VRX-300 respectively. Chemical shifts are given in ppm relative to that of Me4Si (δ = 0) in CDCl3. The abbreviations of signal patterns are as follows: s, singlet; d, doublet; t, triplet; m, multiplet. Mass spectra were obtained with a HP5985-B spectrometer. IR spectra were recorded on a Perkin-Elmer 200s spectrometer. (1, 2, 6, 73H) Testosterone [3H]J, specific activity: 95 Ci/mmol and (1, 2, 4, 5, 73H) dihydrotestosterone [3H]J, specific activity 110 Ci/mmol, were provided by New England Nuclear Corp. (Boston, MA, U.S.A.). Radiolabeled T and 5-DHT were supplied by Steraloids (Wilton, NH, U.S.A.). Sigma Chemical Co. (St. Louis, MO, U.S.A.) supplied NADPH; glucose-6-phosphate and glucose-6-phosphate dehydrogenase. The finasteride was obtained by extraction from Proscar (Merck, Sharp & Dohme).

**Synthesis of the Steroidal Compounds**

The synthesis of the intermediates 9, 11—15 (Fig. 5) is given in refs. 15 and 16. The preparation of the new compounds 4, 5, 10, 6—8 is briefly described below.

16β-Bromo-17α-hydroxy-17β-cyclopentylacetate-1,4,6-diene-3,20-dione 4

A solution of steroid 10 (1 g, 2.43 mmol) and DDQ (1 g, 13.22 mmol) in dioxane (50 ml) was allowed to reflux for 3 d. Upon cooling, the precipitated 2,3-dichloro-5,6-dicyanohydropyrimidine was filtered off. The filtrate was added 5% aqueous sodium hydroxide solution (100 ml) and chloroform (100 ml); the mixture was stirred for 5 min. The organic phase was separated, washed 3 times with 3% aqueous sodium hydroxide solution and water. It was dried over anhydrous sodium sulfate and the solvent was removed in vacuum. The crude product was purified by silica gel column chromatography. Hexane-ethyl acetate (1 : 1) eluted 404 mg, 0.99 mmol (40% of the pure product, mp 134—135°C. UV (nm) 217, 253, 291 (E 19900). IR (KBr) cm−1: 3375, 1715, 1700, 1603. 1H-NMR (CDCl3) δ: 1.3 (3H, s), 1.4 (3H, s), 1.9 (3H, s), 2.1 (3H, s), 3.5 (1H, s), 6.0 (1H, d, J = 2 Hz). 13C-NMR (CDCl3) δ: 15.8 (C-18), 21.7 (C-19), 28.6 (C-21), 128.0 (C-6), 162.2 (C-5), 186.4 (C-3), 205.4 (C-20), MS (m/z) 404 (M+) (Fig. 4).

16β-Bromo-17α-cyclopentylacetate-1,4,6-diene-3-pregnatriene-20-one 5

A solution containing steroid 4 (1 g, 2.47 mmol), PTSA (100 mg), trfluoroacetic anhydride (2 ml, 9.53 mmol) and cyclopentylacetic acid (0.8 ml, 4.99 mmol) was stirred for 2 h at room temperature (nitrogen atmosphere). The reaction mixture was neutralized with an aqueous sodium bicarbonate solution to a pH of 7 and diluted with chloroform (10 ml). The organic phase was separated, washed with water and dried over anhydrous sodium sulfate; the solvent was eliminated in vacuum. The crude product was purified by silica gel column chromatography. Hexane-ethyl acetate (1 : 1) eluted 336 mg, 0.74 mmol (30%) of the pure product 5, mp 134—136°C. UV (nm) 217, 253, 291 (E 14600, 12400, 17500 respectively). IR (KBr) cm−1: 3731, 1710, 1656, 1603. 1H-NMR (CDCl3) δ: 1.1 (3H, s), 1.3 (3H, s), 2.1 (3H, s), 5.8 (1H, d, J = 2 Hz). 13C-NMR (CDCl3) δ: 15.5 (C-18), 20.2 (C-19), 33.3 (C-21), 168.1 (C-5), 175.4 (ester carbonyl), 186.1 (C-3), 199.5 (C-20). MS (m/z) 500 (M+).

16β-Bromo-3β,17α-dihydroxy-1β,4,6-diene-5-one-ene-20-one 10

A solution containing steroid 9 (1 g, 3.03 mmol) and hydrobromic acid (5.8 ml) in dioxiane (12.2 ml) was stirred for 6 h at room temperature. The reaction mixture was poured into a beaker containing 30 ml of water. The resulting precipitate was filtered off and washed with water. The crude product was recrystallized from ethyl acetate–hexane; yield 0.72 g, 1.77 mmol (58%) of the pure product 10, mp 196—198°C. IR (KBr) cm−1: 3375, 1700, 1048. 1H-NMR (CDCl3) δ: 1.0 (3H, s), 1.2 (3H, s), 2.4 (3H, s), 3.5 (1H, m), 4.0 (1H, d, J = 2 Hz). 13C-NMR (CDCl3) δ: 16.2 (C-18), 19.3 (C-19), 28.2 (C-21), 71.6 (C-5), 140.8 (C-3), 120.9 (C-6), 51.7 (C-16), 206.4 (C-20). MS (m/z) 340 (M+).

17α-Cyclopentylacetone-16β-methylpregna-4,6-diene-3,20-dione 6

A solution containing steroid 15 (1 g, 2.8 mmol), PTSA (200 mg), trfluoroacetic anhydride (0.2 g, 1.04 mmol) and cyclopentanecarboxylic acid (1.2 ml, 8.7 mmol) was stirred for 2 h at room temperature (nitrogen atmosphere). The reaction mixture was neutralized with an aqueous sodium bicarbonate solution to pH of 7 and diluted with chloroform (10 ml). The organic phase was separated, washed with water and dried over anhydrous sodium sulfate; the solvent was eliminated in vacuum. The crude product was purified by silica gel column chromatography. Hexane-ethyl acetate (8.2) eluted 850 mg, 1.74 mmol (62%) of pure product 6, mp 182—183°C. UV (nm) 283 (ε 22900). IR (KBr) cm−1: 1724, 1666, 1625. 1H-NMR (CDCl3) δ: 0.81 (3H, s), 1.1 (3H, s), 2.0 (3H, s), 2.4 (3H, s), 3.5 (1H, m), 4.0 (1H, d, J = 2 Hz). 13C-NMR (CDCl3) δ: 16.2 (C-18), 19.3 (C-19), 28.2 (C-21), 71.6 (C-5), 140.8 (C-3), 120.9 (C-6), 51.7 (C-16), 206.4 (C-20). MS (m/z) 410 (M+).

17α-Cyclopentylcarbonyle-16β-methylpregna-4,6-diene-3,20-dione 7

A solution containing steroid 15 (1 g, 2.21 mmol), PTSA (200 mg), trfluoroacetic anhydride (2.1 g, 1.04 mmol) and cyclopentanecarboxylic acid
Agonist (800 g) were centrifuged at 14000×g for 1 h at 4 °C in a SW 60 Ti rotor (Beckman Instruments, Palo Alto, California). Cytosol protein content was determined by Bradford's dye binding method(25) using bovine serum albumin (BSA) as the standard.

Androgen Receptor Binding Assay

Assays were performed essentially as described by Cabeza et al.16

Saturation Curves and Determination of the Binding Parameters

Binding sites were saturated in the presence of increasing concentrations (0.5—2.5 μM) of [3H]DHT. Results were analyzed with Scatchard plots,(14) the calculation of affinity (Kd) and the total number of sites (Bmax) were determined by linear regression.

Aliquots of 120 μl of cytosol (2.64 mg of protein) were added and incubated (in duplicate) for 18—20 h at 4 °C and 10% glycerol (v/v). The mixture was incubated for 45 min at 4 °C. The tubes were vortexed and immediately centrifuged at 800×g for 10 min.

Competition Analysis of Synthesized Steroids for ARs

The competition analysis of steroids 4—8 for the androgen receptors of castrated male hamster seminal vesicles was determined on 58 animals (150—200 g), selected at random and gonadectomized 30 d before the experiments. 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.

Androgen receptor competition studies were performed essentially as described by Cabeza et al.13 and are briefly summarized below.

Competition Studies for Competition analysis of steroids, tubes contained 1.26 nm [3H]DHT (specific activity 110 Ci/mmol) plus a range of increasing concentrations (50—500 μM) of the following unlabeled steroid hormones: 5α-DHT, and 4—8 were prepared.

Aliquots 120 μl of cytosol (2.64 mg of protein) were added and incubated (in duplicate) for 18—20 h at 4 °C in tubes mentioned above. Parallel sets of tubes containing identical concentrations of [3H]DHT plus 100-fold excess of radioinert DHT were also incubated. Dextran coated charcoal (800 μl) in TEDAM buffer was added and the mixture was incubated for 45 min at 4 °C. The tubes were vortexed and immediately centrifuged at 800×g for 10 min.

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