Synthesis and Pharmacological Evaluation of New Progesterone Esters as 5α-Reductase Inhibitors

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In this study we report the synthesis and pharmacological evaluation of four new progesterone derivatives; 17α-hydroxy-16β-methylpregna-4,6-diene-3,20-dione 12, 17α-cyclopropylcarbonyloxy-16β-methylpregna-4,6-diene-3,20-dione 13, 17α-cyclobutylcarbonyloxy-16β-methylpregna-4,6-diene-3,20-dione 14, and 17α-acetoxy-16β-methylpregna-4,6-diene-3,20-dione 15 and the pregnatriene compound 17α-cyclobutylcarbonyloxy-16β-methylpregna-1,4,6-triene-3,20-dione 16. The pharmacological effect of these compounds was determined in vitro as well as in vivo. The evaluation in vivo was carried out on gonadectomized male hamsters that were injected subcutaneously daily with testosterone (T) and/or finasteride, or with the novel compounds. At the end of the treatments the animals were sacrificed and the prostates were weighed. It was observed that when testosterone (T) and finasteride or compounds 12—16 were injected together, the weight of the prostate decreased significantly as compared to that of the testosterone-treated animals. The 5α-reductase inhibitory activity was evaluated in vitro using human prostate homogenates. These experiments showed the following IC50 values: compound 12 (alcohol at C-17) 1.2×10−6 M, 13 (cyclopropyl substituent at C-17) 7.9×10−10 M, 14 (cyclobutyl substituent) 6.3×10−11 M and 16 (cyclobutyl substituent) 3.9×10−4 M. It is evident from these data that when the size of the substituent at C-17 is decreased, the 5α-reductase inhibitory activity increases. Apparently, in this biological model, the 5α-reductase inhibitory activity depends upon the steric effect of the substituent at C-17. However, the free alcohol 12 showed much lower 5α-reductase inhibitory activity.

Key words 5α-reductase; dihydrotestosterone; antiandrogen; seminal vesicle; prostate

Steroid 5α-reductase is a NADPH-dependent enzyme responsible for the reduction of testosterone (T) 1 into the more potent androgen (DHT) 2. This enzyme is located in the androgen-dependent tissue such as the prostate, seminal vesicles, epididymis and other reproductive tissues.1 It has been determined that DHT 2 interacts more efficiently with the androgen receptors than its precursor T 1 and has been implicated in the pathogenesis of prostate cancer, benign prostatic hyperplasia (BPH), acne and male pattern baldness.2,3 This fact indicates that both the 5α-reductase enzyme and DHT 2 play important physiological and pathological roles in human males. Therefore, the inhibition of DHT formation by the 5α-reductase enzyme is a logical treatment for androgen dependent afflictions.4

Recently, several new inhibitors of 5α-reductase were described as potential clinical candidates for the treatment of androgen-dependent disorders. These compounds (Fig. 1) include finasteride 3, turosteride 4 and dutasteride 5. Finasteride 3, a 4-azasteroid is the first 5α-reductase inhibitor approved in the U.S.A. for the treatment of BPH. In humans, finasteride decreases prostatic DHT levels by 70—90% and reduces prostate size, while T tissue levels remain constant.5 The use of finasteride demonstrated a sustained improvement in the treatment of androgen dependent diseases, and it also reduces the prostate specific antigen (PSA) levels.6,7 Several years ago, we synthesized several new 17α-acyloyloxypregnadiene and pregnatrienes derivatives that showed a high inhibitory effect for the enzyme 5α-reductase as well as high affinity for androgen receptors.8,9 In view of the fact that endocyclic dienes and trienes described in these references8,9 showed high biological activity, in this paper we report the synthesis and pharmacological evaluation of five (12—16, Fig. 3) new similar compounds, based on the progesterone skeleton, as inhibitors for the hamster and human 5α-reductase enzyme. The in vitro effect was assayed in human prostate,10 and the in vivo effects were determined in the prostate of gonadectomized male hamsters.11,12

Results

Synthesis of New Compounds Compounds 12—16 (Fig. 3) were prepared from the commercially available 16-dehydropregnenolone acetate 6 (Fig. 2). Esterification of the hydroxy group in 12 with trifluoroacetic anhydride, the corresponding carboxylic acid and PTSA yielded the desired esters 13—16. Treatment of 14 with 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ) in dioxane afforded the triene-dione 16.

In Vivo Experiments After castration, the weight of the

Fig. 1. Steroidal Compounds

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male hamster prostate 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 for 6 d significantly increased ($p<0.005$) the weight of this gland in castrated male hamsters (Table 1). When testosterone (T) and finasteride or compounds 12—16 were injected together, the weight of the prostate decreased ($p<0.005$) as compared to that of testosterone-treated animals (Table 1).

**In Vitro Experiments** The *in vitro* biological activity of compounds 12—16 was determined in human prostate gland which was homogenized and centrifuged to obtain the prostatic enzyme fraction. The activity of human 5α-reductase was assessed by incubating the enzymatic fractions with 2 nM [3H]T. The dichloromethane extracts from human prostates were subjected to TLC analysis. The zone corresponding to the DHT standard ($R_f$ value of 0.67) of the experimental chromatogram was cut off, soaked in Ultima Gold, and the radioactivity determined. This result was considered to be 100% of the activity of 5α-reductase for the development of inhibition plots. Unmodified [3H]T was identified from control incubations which did not contain tissue.

**Determination of 50% Inhibitory Concentration of the New Compounds in Human Prostate** The concentrations of compounds 3 and 12—16 required for inhibiting 5α-re-

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Prostate weight (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>44.8±7.9</td>
</tr>
<tr>
<td>T</td>
<td>81.6±6.6</td>
</tr>
<tr>
<td>T+3</td>
<td>39.7±3.2</td>
</tr>
<tr>
<td>T+12</td>
<td>75.0±7.6</td>
</tr>
<tr>
<td>T+13</td>
<td>63.9±4.4</td>
</tr>
<tr>
<td>T+14</td>
<td>41.8±7.8</td>
</tr>
<tr>
<td>T+15</td>
<td>71.3±13.7</td>
</tr>
<tr>
<td>T+16</td>
<td>63.9±4.4</td>
</tr>
</tbody>
</table>
The IC₅₀ values were determined for Finasteride and the synthesized steroids 12—16 with human prostate 5α-reductase activity by 50% (IC₅₀) were determined from the inhibition curves using different concentrations of the steroids, and are shown in Table 2. The IC₅₀ values obtained for compounds 15 (6.3×10⁻¹¹ M), 13 (IC₅₀ 7.9×10⁻¹⁸ M) and for finasteride 3 (IC₅₀ of 8.5×10⁻⁹ M) were lower than those for 12 (IC₅₀ of 1.2×10⁻⁶ M), 14 (IC₅₀ of 3.2×10⁻⁸ M) and 16 (IC₅₀ of 3.9×10⁻⁹ M), (Table 2).

**Table 2: The IC₅₀ Values Were Determined for Finasteride and the Synthesized Steroids 12—16 with Human Prostate 5α-Reductase**

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IC₅₀ value (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Finasteride 3</td>
<td>8.5×10⁻⁹</td>
</tr>
<tr>
<td>12</td>
<td>1.2×10⁻⁶</td>
</tr>
<tr>
<td>13</td>
<td>7.9×10⁻¹⁰</td>
</tr>
<tr>
<td>14</td>
<td>3.2×10⁻⁸</td>
</tr>
<tr>
<td>15</td>
<td>6.3×10⁻¹¹</td>
</tr>
<tr>
<td>16</td>
<td>3.9×10⁻⁹</td>
</tr>
</tbody>
</table>

They represent the concentration of the steroid that inhibits 50% of 5α-reductase activity, and were determined as described in Experimental.

**Discussion**

This study reports the synthesis and pharmacological evaluation of four pregnadiene derivatives (12—15) and one pregnenolone compound 16. As can be seen in Table 1, all derivatives decreased the weight of the prostate gland compared to that of proscar 3 (wt of the prostate gland 41.8 mg) as compared to the steroidal esters 12 (IC₅₀ of 1.2×10⁻⁶ M), 14 (IC₅₀ of 3.2×10⁻⁸ M) and 16 (IC₅₀ of 3.9×10⁻⁹ M), (Table 2).

**Experimental**

**Chemical and Radioactive Materials** 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). Mass spectra were obtained with an HP5985-B spectrometer. IR spectra were recorded on a Perkin-Elmer 200s spectrometer. (1.2,6,7-H) Testosterone [HJT specific activity: 95 Ci/mmol was provided by New England Nuclear Corp. (Boston, MA, U.S.A.). Radiolint T and 5α-dihydrotestosterone were supplied by Steraloids (Wilton, NH, U.S.A.), Sigma Chemical Co. (St. Louis, MO, U.S.A.) supplied NADPH. Finasteride was obtained by extraction from Proscar (Merck, Sharp & Dohme). The tablets were crushed, extracted with chloroform, and the solvent was eliminated in a vacuum; the crude product was purified by silica gel column chromatography.

**Synthesis of the Steroidal Derivatives** Compounds 7—12 (Fig. 2) and 13—16 (Fig. 3) were prepared from the commercially available 16-dehydropregnenolone acetate 6. Epoxidation of the double bond at C-16 in 6 with hydrogen peroxide and sodium hydroxide afforded the epoxy derivative 7. Acetylation of 7 in the usual manner yielded the acetoxoy compound 8. Protection of the carbonyl group in 9 was effected with ethylene glycol, trimethyl orthofornate and p-toluenesulfonic acid (PTSA). The resulting dioxolane derivative 9 was allowed to reflux with methylmagnesium chloride in tetrahydrofuran (THF); this reaction afforded the 16-methyl substituted dioxolane derivative 10. Hydrolysis of the dioxolane ring in 10 to recover the carbonyl moiety at C-20 was carried out with PTSA in acetone, thus forming the carboxylic derivative 11. The oxidation of the hydroxyl group at C-3 in 11 was effected with lithium carbonate, lithium bromide and boron in N,N-dimethylformamide (DMF). Esterification of the hydroxy group in 12 was carried out with trifluoroacetic anhydride, the corresponding acetyl chloride and PTSA; this reaction yielded the desired esters 13—15 (Fig. 3). Treatment of 14 with 2,3-dichloro-5,6-dicyanohydroquinone (DDQ) in dioxane afforded the trienedione compound 16.

Synthesis of the intermediates 7—12 (Fig. 2) is given in refs. 8 and 9. The preparation of compounds 13—16 is briefly described below. The new esters 13—16 (Fig. 3) were prepared according to the following general procedure. A solution containing steroid 12 (1 g, 2.92 mmol), PTSA (0.5 mg), trifluoroacetic anhydride (1.84 g, 8.76 mmol) and the corresponding acid (9 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, then diluted with chloroform (10 ml). The organic phase was separated, washed with water and dried over anhydrous sodium sulfate; the solvent was eliminated in a vacuum. The crude product was purified by silica gel column chromatography. Hexane–ethyl acetate (8:2) eluted the pure ester.
(m/z) 422 (M⁺).

Animals and Tissues Adult male golden hamsters (150—200 g) were obtained from the Metropolitan University in Xochimilco, Mexico. Gonadectomies were performed under pentobarbital anesthesia 30 d prior to the experiments, and the animals were sacrificed with CO₂. This protocol was approved by the Institutional Care and Use Committee of the Metropolitan University of Mexico (UAM). The experiment was comprised of 9 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.

Human prostate from a cadaver was kindly provided by Dr. Avissai Alcántara from the Department of Pathology of The General Hospital (SS) in Mexico City, and stored at −70 °C. Frozen human prostate was thawed on ice and minced with scissors. Unless specified, the following procedures were carried out at 4 °C. Tissue was homogenized with a tissue homogenizer (model 985-370; variable speed 5000—30000 rpm, Biospec Products, Inc.).

In Vivo Experiments For the daily subcutaneous injections, 400 µg of the steroids 12—16 were dissolved in 200 µl of sesame oil and administered for 6 d, together with 200 µg of testosterone (T). Three groups of animals were kept as a control: one was injected with 200 µl of sesame oil, the second with 200 µg of T, and the third with T plus finasteride for 6 d. After the treatment, the animals were sacrificed by CO₂. Prostates from animals (4) of each group were dissected and weighed. Two separate experiments were performed for each group of steroid treated animals. The results (Table 1) were analyzed using one-way analysis of variance with EPISTAT software.

In Vitro Experiments Human prostate was homogenized in 2 volumes of medium A (20 mM sodium phosphate, pH 6.5, containing 0.32 M sucrose, 0.1 mM dithiothreitol Sigma-Aldrich, Inc.) with a tissue homogenizer. Homogenates were centrifuged at 15000×g for 20 min(10) in a SW 60 Ti rotor (Beckman Instruments, Palo Alto, Cal., U.S.A.). The pellets were separated, washed with 3 volumes of medium A and centrifuged two additional times at 44000×g at 0 °C for 10 min.(11) The washed pellets were suspended in medium A and kept at −70 °C. The suspension (5 mg/ml for human prostates), determined by the Bradford method,(12) was used as a source of 5α-reductase.

Determination of 5α-Reductase Activity The enzyme 5α-reductase was assayed as previously described.(10) The reaction mixture contained a final volume of 1 ml: 1 mM dithiothreitol, sodium phosphate buffer (40 mM), at pH 6.5 for human prostates,11 2 mM NADPH, and 2 nM [1,2,6,7-3H]T. The reaction in duplicate was started when it was added to the enzymatic fraction (134 µg protein in a volume of 80 µl),(11) incubated at 37 °C for 60 min, and stopped by mixing with 1 ml of dichloromethane; this was considered the end point. Incubation without tissue was used as a control. The fraction of dichloromethane was separated, and the extraction was repeated 4 more times. The extract was evaporated under a nitrogen stream to dryness and suspended on 50 µl of methanol that was spotted on TLC Keiselgel 60 F₂₅₄ plates. T and DHT were used as carriers, and the plates were developed in chloroform-acetone = 9:1. The plates were air-dried and the chromatography was repeated 2 more times. The steroid carriers were detected using a phosphomolybdic acid reagent and a UV lamp (254 nm). The DHT-containing areas were cut off and the strips were soaked in 5 ml of Ultima Gold (Packard), then the radioactivity was counted in a scintillation counter (Packard tri-carb 2100 TR). Control incubations, chromatography separation and identification were carried out in the same way as described above except that these tubes did not contain tissue.

Determination of 50% Inhibitory Concentration of Steroids 12—16 in Human Prostatic 5α-Reductase In order to calculate the IC₅₀ values (the concentration of steroids 12—16 required to inhibit 5α-reductase activity by 50%), six series of tubes containing increasing concentrations of these steroids (10⁻¹—10⁻³ M) were incubated in duplicate, in the presence of: 1 mM of dithiothreitol, 40 mM sodium phosphate buffer pH 6.5 for human; 2 mM NADPH, 2 nM [1,2,6,7-3H]T and 134 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, adding one ml of dichloromethane to stop the reaction. The amount of DHT formed was determined as we detailed above.

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References
10) Hirosumi J., Nakayama O., Fagan T., Sawada K., Chida N., Inami M., Alcántara from the Department of Pathology of The General Hospital (SS) in Mexico City, and stored at −70 °C. Frozen human prostate was thawed on ice and minced with scissors. Unless specified, the following procedures were carried out at 4 °C. Tissue was homogenized with a tissue homogenizer (model 985-370; variable speed 5000—30000 rpm, Biospec Products, Inc.).