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STEROIDAL INHIBITORS OF PROSTATIC 5α-REDUCTASE: STRUCTURE-ACTIVITY RELATIONSHIPS

G. M. DUPUY, K. D. ROBERTS, G. BLEAU and A. CHAPDELAINE
Departments of Medicine, Obstetrics & Gynecology and Biochemistry, University of Montreal, and Maisonneuve-Rosemont Hospital, Montreal, P. Quebec, Canada

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SUMMARY

Several novel synthetic androgen analogs have been evaluated for their *in vitro* inhibitory activity towards 5α-reductase of the rat ventral prostate. The influence of various structural alterations of the steroid molecule on the inhibitory activity was assessed. It was confirmed that the Δ^4-3 keto group is essential. Substituents at C-17 influence the degree of inhibitory activity. The presence of the C-19 methyl group is not essential for activity. Ring A appears to interact with the entire active site of the enzyme. Affinity for the enzyme is enhanced by the addition of a methylene group at C-6. Inhibitory activity is completely lost when a large radical, such as an iodomethylene group, is introduced at C-7.

INTRODUCTION

Dihydrotestosterone (DHT) is a mediator of androgenic effects in several target organs [1] and the enzyme 5α-reductase is the determinant of the intracellular concentration of this steroid within the prostate gland. The importance of this enzyme has led to investigations concerning its subcellular localization [2–6], cofactor requirements [2–8], optimal pH [3–5] and temperature [4–9] as well as the irreversibility of the reaction [10].

The importance of DHT formation in the expression of androgenic activity in certain target organs prompted a study of several "antiandrogens" as possible inhibitors of the synthesis of DHT. Certain steroids are available that can block the formation of DHT, i.e. progesterone [11–15], 17-hydroxyprogesterone [14–15], estrogens [13, 15–18], desoxycorticosterone, androstenedione [14], compound R4414 [19] and 4-androsten-3-one-17β-carboxylic acid [20]. However, high concentrations must be used in order for them to be effective. Other steroids appear to impair the translocation of the DHT-receptor complex, i.e. cyproterone acetate [21], flutamide [22–23], spirolactone [24–25], compound R2956 [26] and BOMT [27] (4′-nitro-3′-trifluoromethylisobutyrylanilide).

The present investigation concerns the *in vitro* effects of several synthetic compounds on the reaction catalysed by 5α-reductase of the rat prostate. The percentage of inhibition was determined for each compound and a comparison was made with regard to their efficacy as inhibitors of DHT formation.

MATERIALS AND METHODS

Reagents

Solvents used were of analytical grade. $NADP^+$, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were purchased from Sigma; the NADPH generating system was prepared as described previously [28].

The radiochemical purity of [4-^{14}C]-testosterone (T) (57.5 mCi/mmole), [1,2-^{3}H]-dihydrotestosterone (44 Ci/mmole) and [1,2-^{3}H]-5α-androstane-3α,17β-diol (3α-diol) (50 Ci/mmole) (New England Nuclear, Boston, Mass.) was verified by column chromatography on Sephadex LH-20 followed by crystallization after the addition of authentic carriers to aliquots of the tracers. [1,2-^{3}H]-5α-androstane-3β,17β-diol (3β-diol) (51 Ci/mmole) (Amersham/Searle) was purified by chromatography on Lipidex-5,000 (Packard Instruments) and its purity was verified by crystallization with authentic carrier. Unlabelled DHT, 3α-diol and 3β-diol (Steraloids Inc., Wilton N.H.) were crystallized from methanol:water and used as carrier steroids. The androgen analogs, designated A to I, whose structures are presented in Fig. 1, were generously provided by Merck & Co., Rahway, New Jersey. Solutions of these compounds were prepared in purified ethanol.

Steroid nomenclature: Compound A: [2′,3′-tetrahydrofuran-2′ → 17-(4-androsten-3-one)] L586.659; Compound B: [2′,3′-tetrahydrofuran-2′ → 17-(5α-androstan-3-one)] L598.229; Compound C: 4-androsten-3-one-17β-carboxylic acid methyl ester L589.170; Compound D: 4-androsten-3-one-17β-carboxylic acid L552.803; Compound E: 5α-androstan-3-one-17β-carboxylic acid methyl ester L629.917; Compound F: 17β-hydroxy-17α-methyl-2-keto-4-androsten-3-one L634.066; Compound G: 17α-methyl-4-androsten-3-one L589.372; Compound H: [2′,3′-tetrahydrofuran-2′ → 17-(4-estren-3-one)] L586.153; Compound I: [2′,3′-tetrahydrofuran-2′ → 17-(6-methylene-4-androsten-3-one)] L612.710; Compound J: [2′,3′-tetrahydrofuran-2′ → 17-(7-Iodomethyl-4-androsten-3-one)] AC8138-20.

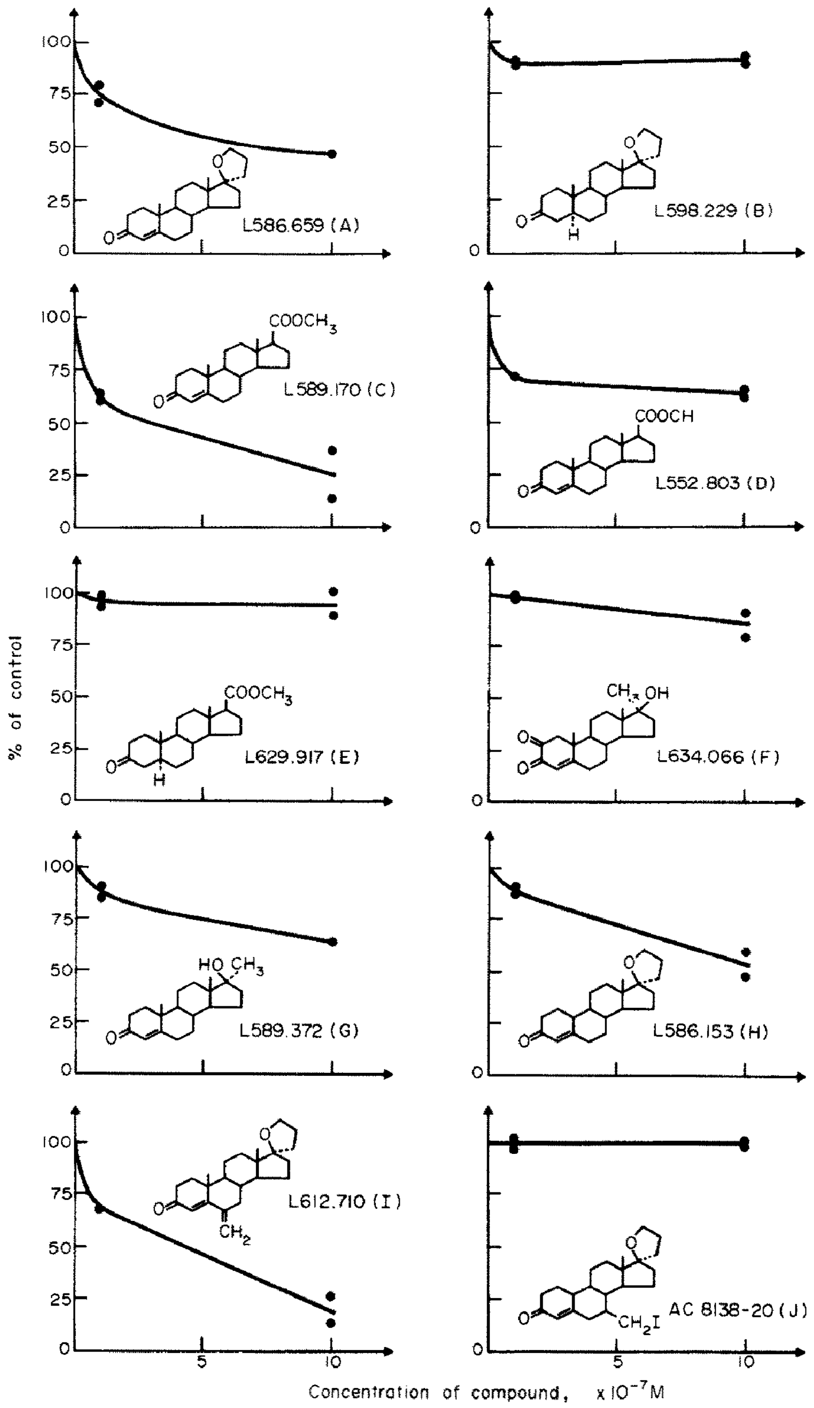

Fig. 1. Effect of different steroid analogs on 5α-reductase activity of rat ventral prostate. The nuclear fraction was incubated with T-^{14}C, 10^{-5} M, in the presence of the different compounds at two concentrations, 10^{-7} and 10^{-6} M under standard conditions. 5α-reductase activity is expressed as percentage of control incubation without analog.

Preparation of tissues

Sprague–Dawley rats weighing between 225 and 250 g were sacrificed by decapitation and the ventral prostates were immediately excised and washed in ice-cold Tris-HCl buffer (Tris 0.05 M, EDTA 1.5 mM, mercaptoethanol 2 mM, pH 7.4). After homogenization with a Potter–Elvejhem apparatus, the crude nuclear fraction was isolated by centrifugation at 900 *g* for 10 min in a Sorvall RC2-B centrifuge. The pellets were washed and this nuclear fraction was used for the enzymatic assays.

Enzyme assay

The substrate, testosterone-[^{14}C], was used at a concentration of 10^{-5} M. The antiandrogens to be studied for their effect on enzyme activity were added to the incubation medium at two different concentrations (10^{-7} M and 10^{-6} M). Propylene glycol (0.05 ml) was added to dissolve the compounds in buffer. The NADPH generating system was added and the reaction was initiated by the addition of the nuclear fraction; the final volume was 5 ml. Incubations were of 30 min duration at 37°C in a Dubnoff metabolic Incubator (Precision Scientific Co., Chicago). Incubations were terminated by the addition of 1 volume of ether. Known amounts of [1,2-^{3}H]-DHT, [1,2-^{3}H]-5α-androstane-3α,17β-diol and [1,2-^{3}H]-5α-androstane-3β,17β-diol were added to each tube in order to account for procedural losses. Unlabelled DHT, 3α-diol and 3β-diol (75 μg) were also added to each tube in order to facilitate their detection.

Three extractions (v/v) were performed with ether purified on aluminum oxide columns (Woelm neutral, Activity 1, Waters Associates Inc., Framingham, Mass.). The ether extracts were combined and evaporated to dryness. The residue was then submitted to paper or thin layer chromatography in the following systems:

I. Benzene:heptane:methanol:water (34:66:80:20) at ambient temperature for 4½ h; support: Whatman paper No. 1.
II. Benzene:ethyl acetate (1:1) 4 h on alumina (20 × 20 cm precoated alumina plates, Eastman Chromatogram Sheet 6062).
III. Benzene:ethanol (95:5), 6 h on alumina t.l.c. plates.

Radioactive products were located by scanning papers or t.l.c. plates with a Packard Radiochromatogram scanner, model 7201. Non-radioactive DHT was located using the Zimmermann reaction (potassium hydroxide 15%, *m*-dinitrobenzene 2%); 5α-androstane-3α,17β-diol and 5α-androstane-3β,17β-diol were localized with an alcoholic solution of phosphomolybdic acid. Following the addition of mg amounts of authentic steroid, each purified metabolite was crystallized from methanol–water to constant ^{3}H/^{14}C ratio. Radioactivity was assayed using a Packard liquid scintillation spectrometer, model 3375. Maximal counting efficiency was 71% for ^{14}C and 41% for ^{3}H using a dual label discriminator setting.

Isolation, purification and quantitative determination of DHT, 5α-androstane-3α,17β-diol and 5α-androstane-3β,17β-diol

DHT. The residue from the ether extract was chromatographed in system I. The area corresponding to the R_F of DHT was eluted from the paper and rechromatographed in system II. DHT was then crystallized to constant ^{3}H/^{14}C ratio with added carrier steroid.

5α-androstane-3β,17β-diol. The residue with an R_F corresponding to this androgen, after chromatography in system A, was eluted and crystallized to constant ^{3}H/^{14}C ratio.

5α-androstane-3α,17β-diol. The radioactive product migrating with 3α-diol in system A was chromatographed in system C and then crystallized to constant ^{3}H/^{14}C ratio following admixture with carrier steroid.

RESULTS

In each of the incubations, the percentage transformation of T to 3β-diol was less than 0.01% and less than 1% to 3α-diol. The total 5α-reductase activity was calculated by addition of the percentages of transformation of T to DHT and 3α-diol.

Duplicate experiments were performed and the results are expressed as the percentage of 5α-reductase activity remaining in the presence of a given inhibitor. The results are illustrated in Fig. 1. Four compounds (A, C, H and I) were potent inhibitors (at least 45% inhibition at 10^{-6} M). It has been already demonstrated that compound A is a potent inhibitor with a K_I of 5×10^{-8} M (28). In the present experiment, this compound produced an inhibition of 53% at a concentration of 10^{-6} M. The other three compounds, used at the same concentration, produced the following percentages of inhibition: Compound C, 76%; Compound H, 47%; and Compound I, 81%. It should be noted that all of these compounds possess a Δ^4-3 keto structure.

A second classification of inhibitors can be made, according to their degree of inhibition, with compounds D and G (more than 25% inhibition). They produce an inhibition of 26% and 37%, respectively, at a concentration of 10^{-6} M. They also bear a Δ^4-3 keto group but differ in their substituents at position 17.

A third classification of inhibitors comprises compounds B, E, F and J which produced less than 25% inhibition of the reaction, i.e. 6%, 6%, 15% and 0%, respectively.

Only compound F, possessing a Δ^4-3 keto structure with an additional 2-keto group resulted in a small but significant decrease of the enzyme activity. It should be noted that compound B and E are 5α-reduced products. Compound J is reduced at position 5α but is substituted at C-7 with a grouping of large molecular size.

DISCUSSION

In our studies concerned with the physiological action of androgens, a search was undertaken for antiandrogens of high potency and specificity. In previous work [28], this fact was also demonstrated since compound A was found to be a potent inhibitor of 5α-reductase (K_I of 5×10^{-8} M) while compound B did not possess any inhibitory activity. It was reported by Voigt and Hsia, who studied inhibitors of 5α-reductase of human skin [20], that the Δ^4-3 keto structure is necessary for effective inhibitory activity. This finding is also supported by the present data since the 5α-reduced compounds B and E are not effective inhibitors. Compound F, possessing a 2-keto group, does not inhibit the enzyme, suggesting an interaction of the entire ring A with the active site of the enzyme. Certain of the synthetic compounds studied (Compounds A, C, D and G) were different structurally only by the substituents attached at position 17. Compound G is similar to testosterone with an additional methyl group at position 17α. It would appear, according to Voigt and Hsia, that a 17α-substituent can diminish the attachment of the steroid to the enzyme and therefore reduce its inhibitory effect. The results obtained with compound G are in agreement with this postulate. The other three compounds with different substituents at position 17 (A, C and D) provide varying degrees of inhibition. It is interesting to note that the esterification of the 17-carboxyl group almost doubles the inhibitory potency.

Compound H, which possesses a structure similar to product A except for the absence of a C-19 methyl group, is as an effective inhibitor as compound A. It is obvious, therefore, that the C-19 methyl group in analog A is not necessary for the inhibitory effect. This finding is in contrast with the data of Voigt and Hsia who obtained a diminished inhibitory effect with compounds lacking a C-19 methyl group in their study of 5α-reductase isolated from microsomes of human skin [20]. However, differences may exist between the microsomal 5α-reductase present in human skin and the nuclear 5α-reductase of the rat ventral prostate.

We observed that the addition of a substituent (a methylene group) at position C-6 enhances the inhibitory effect, since compound I is a more powerful inhibitor than compound A. The affinity of a compound for the catalytic site would appear to be enhanced by the presence of this substituent at C-6. However, if substitution is made at C-7 (compound J) with a halogen of large dimension (CH_2I) the inhibitory activity is lost. Without this iodomethylene substitution, the compound possesses good inhibitory activity, i.e. product H. Therefore, substitution at position 7 (compound J) with a large radical impairs the interaction of the steroid with the enzyme and thereby abolishes its strong inhibitory effect.

These data provide additional information concerning the structural requirements of a steroid for interaction with the active site of the enzyme. In summary, the Δ^4-3 keto structure is obligatory for the attachment of the steroid to the enzyme since 5α-reduced products are ineffective. Substitution at C-2 with a hydrophilic group, almost completely abolishes the inhibitory activity. The compounds with Δ^4-3 keto structure and different substituents at position 17 are all good inhibitors.

It also can be concluded that the C-19 methyl group is not essential for the inhibition and that substitution at C-6 can enhance the inhibitory effect. However, substitution at C-7 with a molecule of large dimension completely abolishes the inhibitory effect.

These conclusions are made with the assumption that the compounds tested act by competition with testosterone for the active site of the enzyme. In this regard, previous kinetic data [28] have clearly demonstrated that compound A is a competitive inhibitor.

The aim of these studies is to use an antiandrogen to block specifically the enzymatic reduction of testosterone. This would allow the identification of the metabolic effects of testosterone vs dihydrotestosterone in cultured prostatic cells. This antiandrogen should be potent and specific without displacing the natural androgens from cellular receptors and studies are in progress in an attempt to find such an analog.

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