IN VITRO BINDING OF 16-METHYLATED C_{18} AND C_{19} STEROID DERIVATIVES TO THE ANDROGEN RECEPTOR

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The binding of androgens and structurally related analogues to the androgen receptor was studied. The *in vitro* experiments were carried out with cytosol of castrated rat prostate, using [^{3}H]R1881 (methyltrienolone) as radioligand. The binding parameters measured were K_d=1.25 $\times 10^{-10}$ M and B_{max}=111 fmol (mg protein)$^{-1}$. Ligand specificity was confirmed by competition experiments with known androgen, oestrogen and progestogen ligands. The receptor binding of substituted steroids was studied. The RBAs (relative binding affinities) of our recently synthetized 16-alkyl steroids were low. The only exception was the 17β-hydroxy-16β-methylestr-4-en-3-one, which exhibited the remarkable RBA of 22.9%.

KEY WORDS: androgen receptor binding, 16-alkyl steroids.

INTRODUCTION

Diversities in the mechanisms of action of steroid hormones have emerged in recent years [1]. The new mechanisms proposed do not ignore the classical action of steroids: they may occur either in conjunction with this, or as an alternative mechanism.

The major mechanism in the classical concept involves steroid hormone binding to the intracellular receptor, activation of the receptor, and binding of the steroid-receptor complex to DNA, followed by gene activation [2].

Besides the direct methods of receptor characterization, indirect means of obtaining information are likewise very important and useful in drug research. Different overlapping aims can be distinguished in the study of ligand–receptor complexes [3]. The design of compounds for use as potential drugs is possibly the most spectacular one. The identification of ligands to be used as probes to detect the steroid receptor is very important from a therapeutical point of view. Comparisons between ligands with similar affinities but different activities and/or specificities, can help towards a better understanding of the molecular mechanism. Study of the structural requirements for ligand binding, and thereby mapping of the hormone-binding site of the receptor, is possible by comparing the structures and relative binding affinities of modified steroids.

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We recently reported the preparation of 16-alkyl steroids and their *in vitro* inhibitory effects on 5α-reductase. We now describe the *in vitro* binding of these compounds to the androgen receptor.

MATERIALS AND METHODS

Steroids

Methyltrienolone (R1881, 17β-hydroxy-17α-methylestra-4,9,11-trien-3-one) (compound 1) and [^{3}H]R1881 (S.A.=86 Ci mmol^{-1}) were obtained from New England Nuclear Research Products (Boston, USA). Unlabelled ligands were purchased from Sigma Chemical Company (St. Louis, USA). The studied compounds that we earlier synthesized are listed in Table I. Their chemical structures are given in Fig. 1. All steroids have been found homogeneous by chromatographic methods. Their preparation and purification just as data on the purity (C, H analysis; ^{1}H and ^{13}C NMR) have been published earlier [4–8].

TEDGM+PMSF buffer

Composition: 50 mM Tris–HCl (2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride), 1 mM EDTA (ethylenediaminetetraacetic acid), 1 mM DTT (dithiothreitol), 10% glycerol, 10 mM Na_2MoO_4, 1 mM PMSF (phenylmethylsulfonyl fluoride) and 0.02% NaN_3 (pH: 7.4).

Dextran coated charcoal (DCC)

Three hundred and thirty three milligrams of Norit-A (activated carbon) and 33 mg dextran T-70 in

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100 ml TEDGM+PMSF buffer; always freshly prepared.

Saturation experiments

Prostates of Wistar rats (240–320 g) were removed surgically 24 h after castration. Homogenization was performed in TEDGM+PMSF buffer (mass four times the mass of prostate tissue) with an Ultra-Turrax (Janke and Kunkel, Germany). The homogenate was centrifuged at 4°C, with 18 000 *g* for 30 min.

The protein content of the post-mitochondrial supernatant (PMS) was determined according to Lowry *et al.* [9].

Varying concentrations (from 0.1 nM up to 2.0 nM) of the ligand [^{3}H]R1881 were added to 0.1 ml PMS sample, which was then diluted to 0.4 ml. After mixing, it was incubated for 18 h at 4°C. The unbound radioactivity was removed by centrifugation after the addition of 0.6 ml charcoal suspension. The radioactivity of the supernatant gave the total ligand binding (T). The non-specific binding (NSB) was measured in a parallel assay tube in the presence of excess unlabelled ligand (400 nM R1881). The specific binding of the ligand is the difference between the total binding and the nonspecific binding. These values are expressed in terms of femtomoles per milligram of protein.

Scatchard transformation of the specific binding data resulted in a linear plot. B_{max} (the amount of binding observed at the saturating concentration of radioligand) and K_d (the binding equilibrium constant, i.e. the concentration at which half the receptor sites are occupied) were obtained by linear regression analysis.

Competition experiments

In competition experiments (Fig. 2), 2 nM [^{3}H]R1881 was incubated in the presence of various concentrations of the inhibitor. The abilities of compounds to inhibit the specific binding of the radioligand are characterized quantitatively by their IC_{50} values (the concentration of inhibitor at which 50% of the specific radioligand binding is inhibited). IC_{50} values were determined by linear regression analysis from logit-log plots. Relative binding affinities are defined by

$$RBA = [IC_{50}([^3H]R1881)/IC_{50}(\text{inhibitor})] \times 100$$

RESULTS AND DISCUSSION

The binding energy regarding the steroid hormone receptors is around −12 to −15 kcal mol^{-1}, corresponding to a dissociation constant of 10^{-9}–10^{-11} M. The H-bonds at the C-3 and C-17 ends of the molecule supply one-third to one-half of this energy, while the van der Waals interactions provide the remainder.

For example, the mean intermolecular van der Waals energy is around −3 kcal mol^{-1} for a suitably located methyl group. On that account many methyl-substituted steroids with the functional groups in different positions (e.g. at C-2, C-6, C-7 or C-17) were synthesized and studied [10]. Among the drugs interacting with the androgen receptor are several which contain a methyl or ethyl function, e.g. oxendolone (Takeda, ethyl at C-16), megestrol acetate (Syntex, methyl at C-6) and rosterolone (Schering, methyl at C-1, propyl at C-17).

Besides the nature of the substituent, the 3D structure of the whole molecule may be crucial as regards the biological activity. The conformation of the sterane skeleton can play an important role, and thus small changes in steroid conformation can modify the affinity and specificity [11]. Further, the stereoisomers of a given compound usually differ greatly in their biological properties (both dynamic and kinetic properties) [12]. Chiral drugs and their ethical and

Table I
List of compounds studied

Structure no. (in Fig. 1)	*Compound*	*Synthesis*
4	17β-hydroxy-16β-methylandrost-4-en-3-one	ref. 4
5	17β-hydroxy-16α-methylandrost-4-en-3-one	ref. 4
6	17β-hydroxy-16,16-dimethylandrost-4-en-3-one	ref. 5
7	16β,17β-epoxymethyleneandrost-4-en-3-one	ref. 8
9	17α-hydroxy-16β-methylandrost-4-en-3-one	ref. 4
10	17α-hydroxy-16α-methylandrost-4-en-3-one	ref. 4
12	17β-hydroxy-16β-methylestr-4-en-3-one	ref. 7
13	17β-hydroxy-16,16-dimethylestr-4-en-3-one	ref. 6
14	16,16-dimethyl-3-oxoestr-4-en-17β-yl acetate	ref. 6
15	17α-hydroxyestr-4-en-3-one (nor-epitestosterone)	ref. 8
16	17α-hydroxy-16β-methylestr-4-en-3-one	ref. 7
17	17α-hydroxy-16α-methylestr-4-en-3-one	ref. 7
20	17β-hydroxy-16,16-dimethyl-5α-androstan-3-one	ref. 5
22	16β-ethyl-5α-androstane-3β,17β-diol	
23	16,16-dimethyl-5α-androstane-3β,17β-diol	ref.5

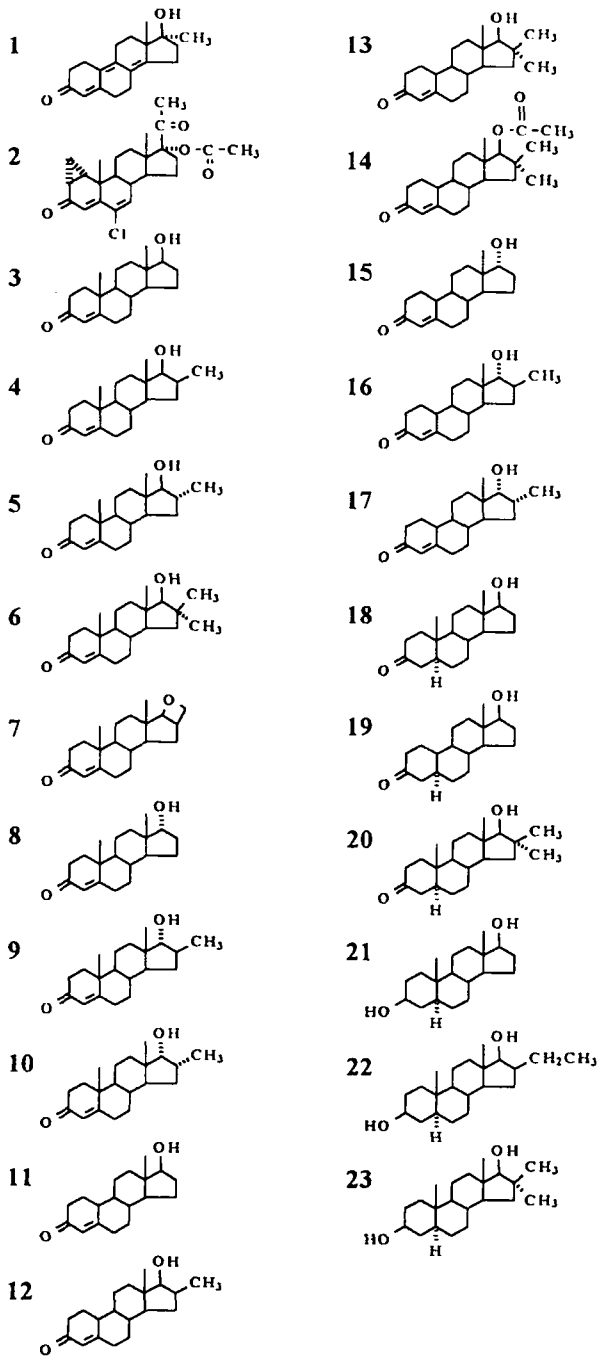

Fig. 1. Chemical structures of compounds studied.

regulatory aspects have been at the focus of development and marketing for many years [13].

Both of the above aspects are involved in our studies of the receptor binding of different, pure isomers of 16-alkylated steroids to the androgen receptor.

Tritiated natural steroid hormones are usually applied as ligands in binding experiments. In the case of the androgen receptor, the synthetic ligand [^{3}H]R1881 is widely used as its affinity is higher than that of tritiated dihydrotestosterone. It does not bind to sex-hormone-binding globulin and it is not metabolized during incubation [14–16]. As R1881 is not a ligand specific for the androgen receptor, but binds

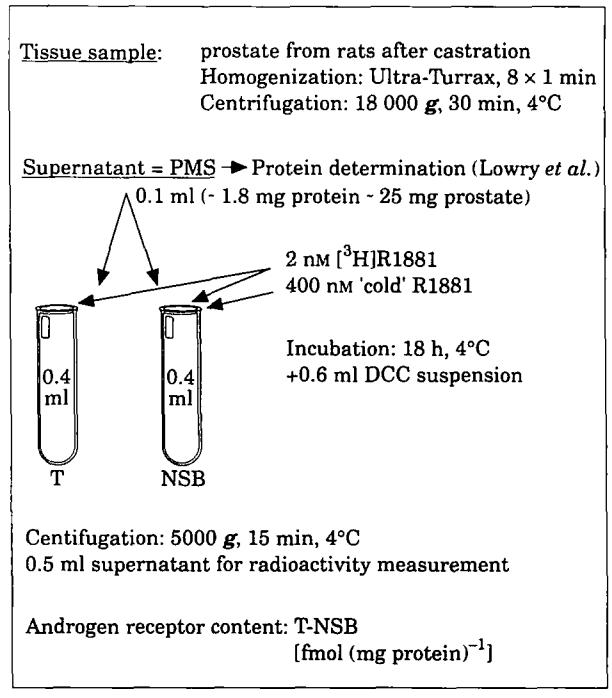

Fig. 2. Assay for androgen receptor binding in cytosol of rat prostate.

with comparably high affinity to the progesterone receptor too, it is possible to study both receptors simultaneously [17] in human tissues. The rat prostate does not contain the progesterone receptor. This permits the use of a simple assay method.

The prostate gland is one of the major androgen-dependent organs. The androgen receptor content of the castrated rat prostate is higher than that of human BPH (benign prostate hyperplasia) prostate cytosol. The average receptor content for different animal groups was found to be 94.8±3.9 fmol (mg protein)$^{-1}$, while that for the human BPH prostate varied in the interval 21-38 fmol (mg protein)$^{-1}$. The rat prostate is therefore commonly applied in androgen receptor binding experiments.

Receptor–ligand interactions have been characterized by the values of B_{max} and K_d. The binding of the ligand [^{3}H]R1881 to the androgen receptor is saturable (Fig. 3), indicating a finite number of binding sites, B_{max}=111 fmol (mg protein)$^{-1}$. The binding affinity meets the criterion of the nanomolar range for receptor identification, with K_d=1.25×10^{-10} M.

The results of competition experiments characterize the pharmacology of the receptor. The data in Table II confirm the ligand specificity. Neither oestrogen nor progestogen-type ligand display binding.

Most of the compounds investigated (structurally related analogues of the natural androgens) exhibit little or no receptor binding. The only noteworthy exception is 17β-hydroxy-16β-methylestr-4-en-3-one (compound 12). Exact values of IC_{50} and RBA are given in Table III.

The basic importance of the 17β-hydroxy-3-one

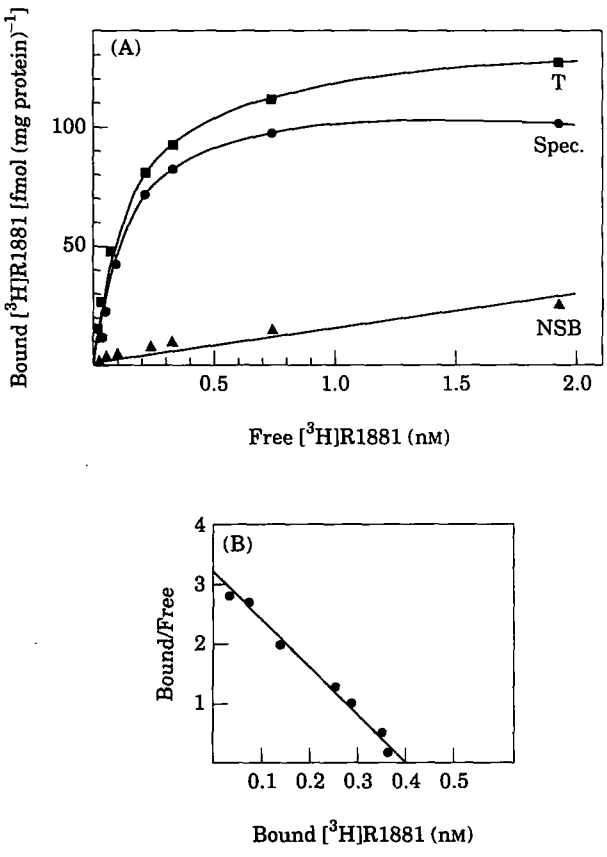

Fig. 3. (A) Saturation curve and (B) Scatchard plot of [^{3}H]R1881 binding in rat prostatic PMS.

structure is confirmed by compound 7. The 16β, 17β-epoxymethylene function results in a loss of binding affinity. The introduction of a 16β-methyl substituent into testosterone (compound 3 → compound 4) decreases the binding from 42.80 to 3.90. The 16α-methyl isomer (compound 5) and the gem-dimethyl analog (compound 6) are practically not bound to the androgen receptor. The 16β-methyl group decreases the binding of 19-nortestosterone too, but to a much lower extent, from 92.20 (compound 11) to 22.90 (compound 12). The consequence of removing the C-10 methyl group from testosterone is a binding increase, from 42.80 (compound 3) to 92.20 (compound 11). A similar effect can be observed for 16β-methyltestosterone (compound 4). Elimination of the C-10 methyl group enhances the RBA from 3.90 (compound 4) to 22.90 (compound 12). It is known from X-ray crystallographic data [18] and molecular geometry calculations [19, 20] that ring A of 19-nortestosterone undergoes a 'flip-flap' interconversion to a half-chair conformation. This interconversion to the very different half-chair conformation explains the

Table III
Relative binding affinities of substituted steroids at 4°C for cytosol androgen receptor of rat prostate in presence of [^{3}H]R1881 as radioligand

Compounds tested	*Range of concentration (nM)*	*IC_{50} (nM)±SD*	*RBA (%)*
R1881	1–20	2.86±0.35	100.00
3	2–20	6.68±0.06	42.80
4	10–120	73.5±18.3	3.90
5	200–2000	>1000	<0.30
6	200–2000	>1000	<0.30
7	200–2000	>1000	<0.30
8	200–2000	824±42.9	0.35
9	200–2000	>1000	<0.30
10	200–2000	>1000	<0.30
11	2–20	3.10±0.80	92.20
12	5–50	12.5±1.6	22.90
13	50–2000	>1000	<0.30
14	500–2000	>1000	<0.30
15	200–1000	203.8±49.8	1.40
16	500–2000	848.3±40.2	0.34
17	250–2000	223.8±17.3	1.30
18	2–20	3.0±0.46	95.30
19	5–160	49.8±13.2	5.70
20	50–1000	523.3±40.8	0.50
21	50–800	73.5±8.5	3.90
22	50–2000	>1000	<0.30
23	50–2000	>1000	<0.30

Table II
Relative binding affinities at 4°C for cytosol androgen receptor of rat prostate in presence of [^{3}H]R1881 as radioligand

Ligands	*Range of concentration (nM)*	*IC_{50} (nM)±SD*	*RBA (%)*
Methyltrienolone (compound 1)	1–20	2.68±0.35	100.00
Cyproterone acetate (compound 2)	2–200	148.0±19.1	1.93
Testosterone (compound 3)	2–20	6.68±0.06	42.80
Dihydrotestosterone (compound 19)	2–20	3.00±0.46	95.30
Estradiol-17β	50–1000	294.0±20.3	0.97
Testosterone propionate	50–1000	338±53.3	0.85
Progesterone	50–1000	663±203	0.43
Estrone	50–2000	>1000.0	<0.30
Estriol	50–2000	>1000.0	<0.30
Ketoconazole	50–2000	>1000.0	<0.30

enhanced binding of 16β-methyl-19-nortestosterone (compound 12) as well. The RBA of epi-testosterone (compound 8) is likewise lower than that of nor-epitestosterone (compound 15). However, the α position of the 17-hydroxy group diminishes the binding affinity so much that even the increased value is very low (1.40 for compound 15). All other analogues with a 17α-hydroxy group (compounds 9, 10, 16 and 17) undergo practically no binding. While dihydrotestosterone (compound 18) has an improved affinity of 95.30 as compared with 42.80 for testosterone, further modification (the lack of the angular methyl group or 16-methylation) lead to compounds 19 and 20 with dramatically decreased binding. 16-alkylation (compound 22: β-ethyl, or compound 23: dimethyl) of 5α-androstane-3β, 17β-diol (compound 21) results in a loss of binding ability.

Receptor binding is essential as regards biological activity. Thus, the IC_{50} and RBA values are important data, but they do not reveal the pharmacological type (agonist or antagonist) of the ligands. Additionally the validation of receptor binding requires *in vivo* biological tests to study the correlation of binding with biological dose–response curves.

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