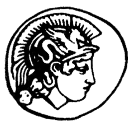

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7α-methyl-19-nortestosterone, a synthetic androgen with high potency: structure-activity comparisons with other androgens

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Abstract

Studies of androgen receptor (AR)-mediated events in vivo are often complicated by problems related to hormone metabolism and pharmacokinetics. Compounds can be metabolically transformed to agents with altered potency. We have investigated some aspects of the structure-activity relationships of testosterone (T) and its analogs using in vivo and in vitro assays. The dose response of ventral prostate (VP) and levator ani (LA) to T, dihydrotestosterone (DHT), 19-nortestosterone (19-NT), 7α-methyl-19-NT (MENT), 7α-cyano-19-NT (CNNT) and 7α-acetylthio-19-NT (ATNT), was investigated in castrated rats. The most potent androgenic steroid (VP response) was MENT followed by T, DHT, 19-NT, ATNT, and CNNT. On the other hand, the order of anabolic potency (LA response) was MENT > 19-NT > T > DHT > ATNT > CNNT. There was a good correlation between bioactivity and binding affinity to AR for the 7α-substituted androgens compared to T. In contrast, relative to their binding affinity to AR, the androgenic potency of DHT and 19-NT was lower compared to T. The reason for the lower in vivo androgenic activity of 19-NT is attributable to its enzymatic conversion to 5α-reduced-19-NT in the prostate. In the case of DHT, the lower bioactivity could be attributed to its faster metabolic clearance rate relative to T. The correlation was further investigated in vitro by co-transfection of rat ARcDNA expression plasmid and a reporter plasmid encoding the chloramphenicol acetyl transferase (CAT) gene driven by an androgen inducible promoter into CV-1 cells. All the androgens led to a dose-dependent increase in the CAT activity. MENT was found to be the most potent followed by DHT, 19-NT, T, and CNNT. The specificity of the androgenic response was confirmed by its inhibition with hydroxyflutamide, an antiandrogen. Thus, there was a good correlation between binding affinity and in vitro bioactivity in the transient transfection assay for the androgens. This suggests that the in vivo bioactivity of androgens could be influenced not only by binding affinity to receptors but also by factors such as absorption, binding to serum proteins and metabolism. However, the high potency of MENT is primarily related to its higher affinity to AR. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Androgens; Biopotency; Receptor binding

1. Introduction

Steroid hormone receptors have pronounced structural requirements for their ligands as shown by specificity of the response and correlation between receptor binding affinity and bioactivity [1–3]. This appreciation led to the development and clinical use of a series of synthetic corticosteroids, progestins and estrogens with higher potency than the natural hormones. However, with respect to androgens, no significant progress has been made in developing high potency compounds. An improvement in therapeutic utilization has been brought about mainly by esterification of testosterone (T) molecule and by depot preparations [4,5]. As early as 1960, marked increase in the androgenic potency was demonstrated for the 7α-methyl derivatives of 19-nortestosterone [6–8]. However, due to weak activity when given orally, no serious attempts were made to utilize these androgens. Avery et al. [9], reported the

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synthesis of 7α-methyl-17aβ-propionyloxy-D-homoestra-4,16, dien-3-one, which showed high androgenic activity parenterally and also exhibited an improvement in oral activity. Our studies have shown that 7α-methyl-19-nortestosterone (MENT), a potent androgen could be a better androgen for hormone replacement therapy and male contraception when given parenterally as sustained release formulations [10–12].

In general, the high potency of 7α-methylated androgens was shown to correlate with their higher binding affinity to androgen receptors [8,13]. However, other reports showed no correlation between the receptor binding affinity and bioactivity of some 7α-methylated androgens [9,14,15]. Thus it was suggested that structural modifications of a compound can lead to changes in its biological activity based on binding affinity, differences in absorption, binding to plasma proteins and/or susceptibility to the action of metabolizing enzymes. The slower metabolism of a synthetic steroid may lead to an increase in its half life in circulation, thus affecting its uptake by target tissues and bioactivity. The activity of a steroid can also be modulated at the level of a target organ by tissue-specific metabolism as is the case with T and 19-nortestosterone (19-NT) [16]. For example, androgenic activity of T is amplified whereas that of 19-NT is reduced upon 5α-reduction in the sex accessory glands. In contrast, their anabolic potency on muscle, where they are not 5α-reduced, is not affected [17].

The present study was undertaken to understand the mechanisms involved in the differences in the biopotency of some of the natural and synthetic androgens. Dose-response curves of their androgenic and anabolic activity in vivo were compared with their relative binding affinities to androgen receptor, sex hormone binding globulin and their in vitro activity in transient transfection assays.

2. Materials and methods

2.1. Reagents

Chemicals and solvents were of reagent grade. 7α-methyl-19-nortestosterone (MENT), 7α-cyano-19-nortestosterone (CNNT) and 7α-acetylthio-19-nortestosterone (ATNT) were custom synthesized by SRI International (Menlo Park, CA, USA). The 7β-methyl-19-nortestosterone was kindly made available by Dr Richard P. Blye, Contraceptive Development Branch, Center for Population Research, NIH, Bethesda, MD, USA. Flutamide was a gift from Dr R. Neri, Schering Plough, Kenilworth, NJ, USA. Testosterone (T), dihydrotestosterone (DHT), and 19-nortestosterone (19-NT) were purchased from Steraloids, Inc., Wilton, NH, USA. The 5α-reduced derivatives of 19-NT, DHNT and MENT, DHMENT were synthesized by Birch reduction over lithium-ammonia solution. The final products were recrystallized in ethyl acetate/hexane to give white crystals of DHMENT (m.p. 205–206°C) and DHNT (m.p. 115–116°C). Molecusol (2-hydroxy-propyl-β-cyclodextrin, 45% aqueous solution) was purchased from Pharmatec Inc., Alchua, FL, USA. Alzet® osmotic pumps were purchased from Alza Corp., Palo Alto, CA, USA.

2.2. Animals and treatment

Immature (21–23 day old) and adult (body weight: 225–275 g) male Sprague–Dawley rats were purchased from Charles River Laboratories, Kingston, NY, USA and housed in accordance with the standards set forth in the NIH Guide for the Care and Use of Laboratory Animals. Rats (6 per group) were castrated through a scrotal incision and randomly distributed into treatment groups. Additional rats were used as intact controls. The dose-response comparisons of 19-NT and MENT with T were done in immature castrated rats. One day following castration, rats were treated sc with different doses of androgens for 10 days. For other dose-response comparisons such as DHT vs T or 7α-cyano/7α-acetylthio-19-NT vs T, adult castrated rats were used. In these studies, treatment was initiated on the day of castration. Androgens were administered by subdermal placement of Alzet® osmotic pumps for 14 days. Androgens were dissolved in Molecusol (45% w/v). At the end of the treatment, rats were exsanguinated,, and the ventral prostate (VP), seminal vesicles (SV), and levator ani (LA) were removed, cleaned of adherent tissue, and weighed.

2.3. Preparation of androgen receptors and determination of binding affinities

One day following castration, male rats (body weight: 300–400 g) were killed and the ventral prostates collected over ice, minced into small pieces and homogenized in 10 mM Tris-HCl buffer, pH 7.4 containing 1.5 mM EDTA, 5 mM dithiothreitol, 10 mM sodium molybdate, 10% glycerol (v/v) and 1 mM phenylmethyl-sulfonyl fluoride (PMSF), using Polytron PT-10 homogenizer. The homogenate was centrifuged for 20 min at 1000 *g* in a refrigerated centrifuge. The supernatant was centrifuged at 105,000 *g* for 90 min at 4°C in a Beckman Ultracentrifuge. The supernatant (cytosol) was removed and stored in aliquots at −70°C until use. Aliquots of cytosol fractions (100–200 μl) were incubated with [^{3}H]-dimethyl-19-nortestosterone (Mibolerone) in the presence of increasing concentrations of competitors at 4°C for 18 h. Bound and free radioactivity was separated by the hydroxylapatite method as described by Isomaa et al. [18]. Hydroxyla-

patite powder (0.67 g) was suspended in 12 ml of separation buffer (10 mM Tris-HCl, 0.5 mM bacitracin, 5 mM dithiothreitol, 10 mM NaH_2PO_4, 10% (v/v) glycerol, pH 7.6. The slurry was mixed repeatedly at 4°C for 30 min, centrifuged at 1500 *g* for 10 min and suspended again in 12 ml of buffer. An aliquot (0.5 ml) of the hydroxylapatite slurry was added to the receptor assay tubes which were vortexed and incubated for 30 min at 4°C with additional vortexing every 5 min. The tubes were centrifuged at 1500 *g* for 10 min, the supernatant was discarded, and 1 ml washing buffer (10 mM Tris-HCl, 5 mM dithiothreitol, 10 mM Na H_2PO_4, 0.25% Triton X-100, 10% glycerol, pH 7.4) was added to each tube. The samples were vortexed three times during the subsequent 10 min incubation and then centrifuged as above. This washing procedure was repeated twice. After the last wash, 1.0 ml ethanol was added to each tube in order to extract the bound radioactivity from the hydroxylapatite pellets. After a 30 min incubation at room temperature, the tubes were centrifuged and the supernatant transferred to counting vials. The pellets were re-extracted with 0.5 ml ethanol, which was combined with the previous extract and counted for radioactivity in 5 ml Beckman Ready Safe® scintillation fluid.

2.4. Binding to sex hormone binding globulin (SHBG)

Third trimester human pregnancy serum was used as a source of SHBG. To remove endogenous steroids, 500 μl of pregnancy serum was treated with DCC (1% Norit-A and 0.5% dextran T-70) in assay buffer (10 mM Tris-HCl containing 10% glycerol 0.2% gelatin, pH 7.4). The solution was incubated for 2 h on ice with intermittent mixing and centrifuged at 4°C for 10 min. The supernatant was kept frozen until used. For the competitive binding studies, an aliquot of the SHBG preparation was diluted 1:100 with assay buffer and duplicate aliquots (100 μl) incubated (18 h at 4°C) with 1 pmol of $[^3H]$-DHT and increasing concentrations of androgens. Bound and free radioactivity was separated by addition of DCC. Supernatant was decanted into scintillation vials containing 5 ml of Beckman Ready Safe® scintillation fluid and counted in a Packard Tricarb scintillation counter. Specific binding was determined by subtracting nonspecific binding from total binding. Specific binding in the presence of unlabeled steroids was plotted as percent relative to control vs molar excess of unlabeled steroids and IC_{50} values were determined.

2.5. Cell culture and transfection assays

CV1 cells (stocks) were maintained in an atmosphere of 10% CO_2 at 37°C in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (Sigma, St Louis, MO, USA) and 1% penicillin — streptomycin (10,000 IU/ml and 10,000 μg/ml) (Flow Laboratories, McLean, VA, USA). For the duration of the transfection experiments, the CV1 cells were maintained in DMEM without red phenol, containing 10% charcoal stripped fetal calf serum.

CV1 cells were plated at a density of 10^6 cell/10 cm dish for 24 h before transfection. The transfections were performed by the calcium phosphate — DNA coprecipitation method according to a procedure described earlier [19]. Typically, each dish was cotransfected with a total of 10 μg DNA containing 4 μg pSG5αAR [20], 4 μg pSMG.CAT (Pharmacia) and 2 μg of lacZ constructs. Cells were incubated with the DNA precipitate for 5 h followed by a 15% glycerol shock for 3 min. Transfection efficiency was monitored with a lacZ expression vector under the control of β-actin promoter. Androgens were added to the cells immediately after transfection. The steroid containing media was replaced 24 h later and the transfected cells were harvested after 48 h.

Chloramphenicol acetyltransferase (CAT) assays were performed as described [21]. Thin-layer plates exposed on X-Omat AR films. CAT activity was quantitated on a Buskin system 200 imaging scanner and normalized to the β-Galactosidase activity. β-Galactosidase activity in the same cellular extracts was determined by using o-nitrophenyl-galactopyranoside (ONPG) as the substrate, essentially as described [22].

3. Results

3.1. Effect of structural modification on binding affinity

3.1.1. Binding to AR

The binding affinity of androgens to AR was assessed using $[^3H]$-Mibolerone as a tracer. The displa-

Table 1
Relative binding affinities of androgens to androgen receptors (AR) and sex hormone binding globulin (SHBG)

Steroid	Relative binding affinity[a]	
	AR	SHBG
1. Testosterone	100 (6.95)	100
2. Nortestosterone (19-NT)	230 (3.01)	5
3. 7α-methyl-19-NT (MENT)	380 (1.82)	6
4. Dihydrotestosterone	290 (2.39)	340
5. Dihydro-19-NT	10 (94.74)	5
6. Dihydro-MENT	30 (24.98)	2

[a] The nanomolar concentration of steroid competitors that reduced binding by 50% were determined. The IC_{50} ratio of the reference compound (testosterone) to the test compound multiplied by 100 is termed the relative binding affinity. The IC_{50} values shown in parentheses were determined from two separate experiments.

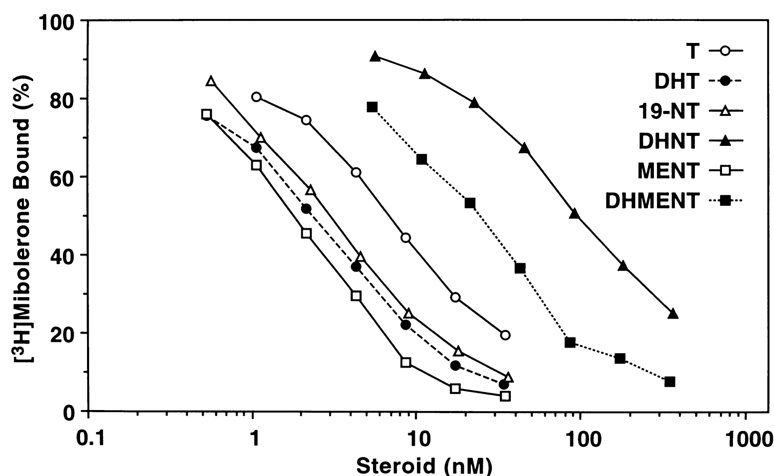

Fig. 1. Competitive inhibition of [^{3}H] Mibolerone binding to androgen receptor (AR), prepared from ventral prostate cytosol, by testosterone (T), dihydrotestosterone (DHT), 19-nortestosterone (19-NT), dihydro-19-NT (DHNT), MENT and dihydro-MENT (DHMENT).

cement curves of different androgens are shown in Fig. 1. A comparison of the relative binding affinities showed that the affinity of MENT (IC_{50} = 1.82 nM) for AR was the highest followed by DHT (2.39), 19-NT (3.01), T (6.95), DHMENT (24.98) and DHNT (94.74) (Table 1). Thus 19-NT had a 2-fold and MENT almost a 4-fold higher RBA than T. However, the 5α-reduced derivatives of both 19-NT (DHNT) and MENT (DHMENT) exhibited much lower RBA compared to the 5α-reduced derivative of T (DHT).

The importance of the substitution with methyl group at 7α-position on 19-NT was investigated by using 7β-methyl, 7α-cyano and 7α-acetylthio substitutions. Methyl substitution at the 7β-position resulted in 8-fold decrease in binding affinity (IC_{50} = 27.3 nM) compared to 7α-methyl derivative (MENT) (Fig. 2). Similarly 7α-substituted cyano or acetylthio 19-NT derivatives showed 10- and 4-fold decrease in binding affinity, respectively, compared to MENT (data not shown).

3.1.2. Binding to SHBG

Since the binding of androgens to SHBG in the circulation is known to affect their clearance, the binding affinity of androgens to SHBG was studied using [^{3}H]-DHT as ligand. The results show that DHT had the maximum ability to displace [^{3}H]-DHT followed by T, MENT, 19-NT, and mibolerone (Fig. 3) (Table 1). The binding affinity of these androgens to SHBG did not correlate with their binding to AR.

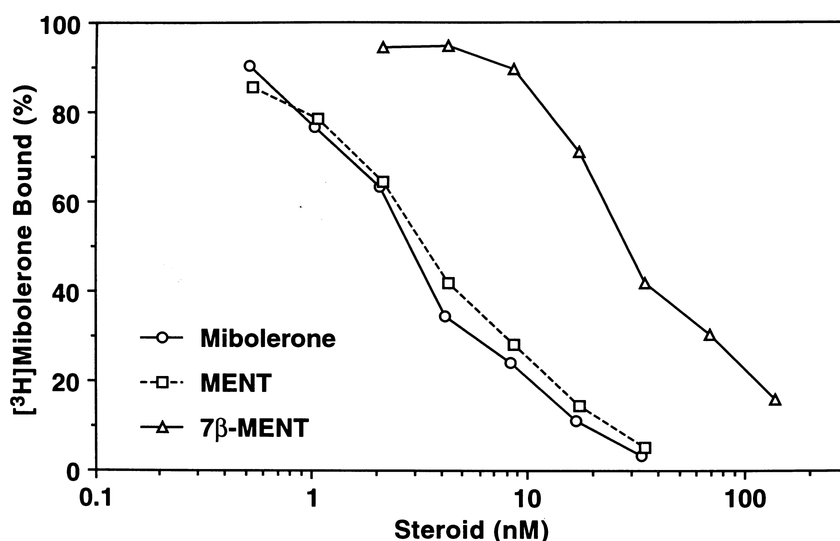

Fig. 2. Competitive inhibition of [^{3}H] Mibolerone binding to AR (ventral prostate cytosol) by mibolerone, 7α-methyl-19-NT (MENT) and 7β-methyl-19-NT (7β-MENT).

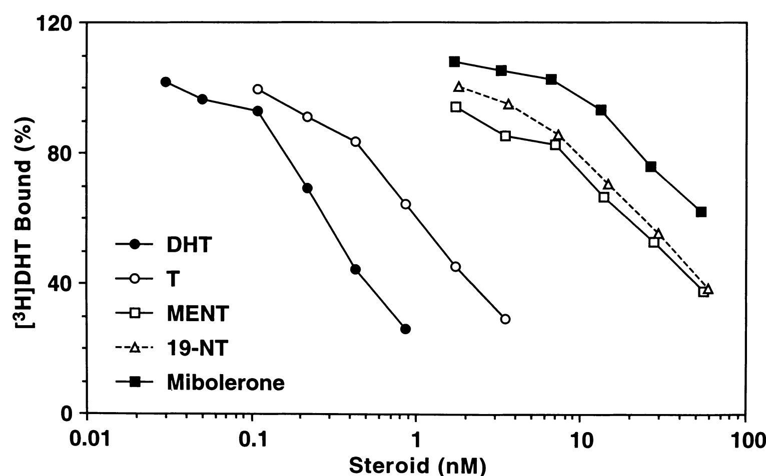

Fig. 3. Competitive inhibition of [^{3}H] DHT binding to Sex Hormone Binding Globulin (SHBG) by different androgens. Third trimester human pregnancy serum was used as a source of SHBG.

3.2. Structure-activity relationships

3.2.1. Androgenic activity in rats

The biopotency of the androgens was investigated in immature castrated rats by the Hershberger assay [23], and adult castrated rats. The dose response comparison of T, 19-NT and MENT in adult castrated rats were reported earlier [24]. Therefore, biopotency of these androgens were compared in immature castrated rats. The dose response curves are shown in Fig. 4. The biopotency of MENT was 4-fold greater than that of testosterone for maintaining VP weights and 15-fold higher for maintaining muscle (levator ani) mass. However, 19-NT, in spite of its greater binding affinity showed only one-fifth the androgenic activity on VP while its anabolic potency was 4-fold greater compared to T. These results of the androgenic/anabolic potency of MENT and 19-NT were comparable to the earlier results in adult castrated rats treated with androgens via Alzet®; osmotic pumps [24]. The androgenic and anabolic potency of DHT was found to be slightly less than that of T, despite its higher RBA (Fig. 5). Thus, as with 19-NT, the androgenic/anabolic potency of DHT did not correlate with its binding affinity to AR in vitro. Since 19-NT could be 5α-reduced to DHNT in the accessory sex organs, the bioactivity of synthetic DHNT was compared with that of 19-NT at equimolar doses in castrated rats. DHNT showed low potency on both VP and LA (Fig. 6), which correlated well with its binding affinity to AR (Table 1). Even though MENT does not undergo enzymatic 5α-reduction, the synthetic 5α-reduced MENT (DHMENT) was found to be only slightly stimulatory to both prostate and muscle (Fig. 6) in agreement with its low binding affinity to AR.

The biological activity of seven substituted 19-NT derivatives was compared in castrated rats. Dose-response comparison of 7α-cyano-19-NT showed it to possess one-fifth the androgenic potency and one half

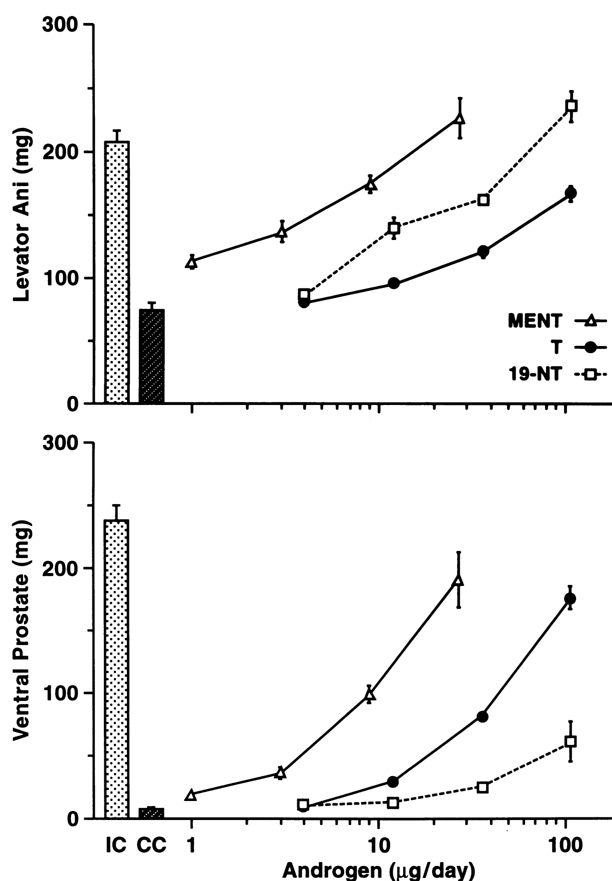

Fig. 4. Dose-response curves for ventral prostate and levator ani weight increases by testosterone (T), 19-nortestosterone (19-NT) and MENT in immature castrated male rats. One day following castration, rats were injected with different doses of the androgens for 10 days. Rats were killed one day after the last injection. IC = intact control; CC = castrated control.

the anabolic potency of T. 7α-acetylthio-19-NT possessed one-half of the androgenic activity of T while its anabolic potency was 2-fold greater (Table 2). Methyl substitution at the 7β position of 19-NT led to almost complete loss of androgenic and anabolic activities (data not shown).

3.2.2. Effect of androgens on CAT activity in transfection assays

The relative potency of the androgens was also compared in co-transfection experiments using CV-1 cells transfected with a rat androgen receptor (AR) expression plasmid and a reporter plasmid encoding the CAT gene under the control of mouse mammary tumor virus long terminal repeat (MMTV-LTR). MMTV-LTR mediated androgen-dependent transcriptional activation of a reporter gene in transiently transfected CV-1 cells [25] that normally do not express endogenous androgen receptors.

The specificity of the response was shown by either omitting the reporter or the expression vector in the DNA-calcium precipitate. In addition, a promoterless reporter construct did not have any CAT activity and an unrelated promoter did not display androgen regu-

Table 2
Comparison of the biopotency of some natural and synthetic androgens on ventral prostate and levator ani[a]

Steroid	Biopotency based on		LA/VP
	Ventral Prostate	Levator Ani	
Testosterone[b]	1.0	1.0	1.0
19-nortestosterone (19-NT)[b]	0.2	4.0	20.0
7α-methyl-19-NT (MENT)[b]	4.0	15.0	4.0
Dihydrotestosterone[c]	0.9	0.7	0.8
7α-cyano-19-NT[d]	0.2	0.4	2.0
7α-acetylthio-19-NT[d]	0.4	2.1	5.0

[a] Potencies were calculated either by 'Allfit' computer program or graphically. The response to testosterone was assigned a value of one.

[b] Biopotency estimates from dose-response comparisons shown in Fig. 4.

[c] Biopotency based on data from Fig. 5.

[d] Biopotencies were calculated from dose-response curves of 7α-cyano-19-NT and 7α- acetylthio-19-NT at four dose levels.

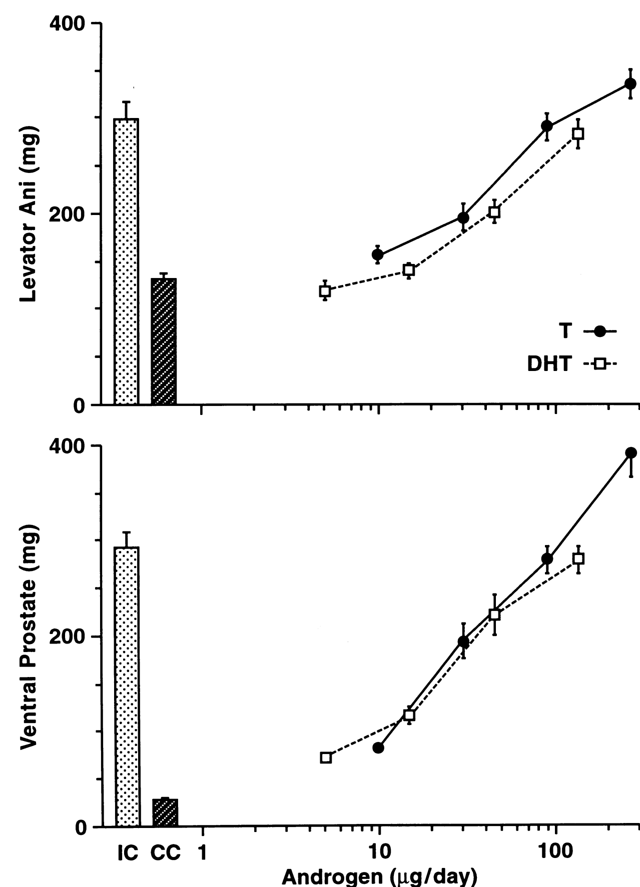

Fig. 5. Dose-response curves for ventral prostate and levator ani weight increases by testosterone (T) and dihydrotestosterone (DHT) in adult castrated rats. On the day of castration, rats were implanted subcutaneously with Alzet osmotic pumps releasing the androgens at different dose levels for 14 days and the rats were killed on day 15.

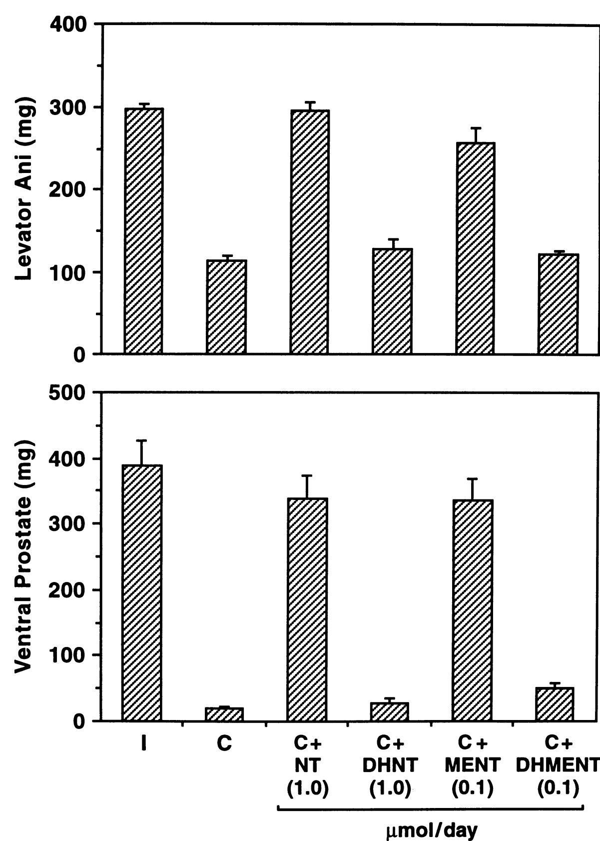

Fig. 6. Effect of 19-nortesosterone (19-NT), MENT and their 5α-reduced derivatives (DHNT and DHMENT) on ventral prostate and levator ani in castrated male rats. Details as per Fig. 5. I = intact; C = castrated.

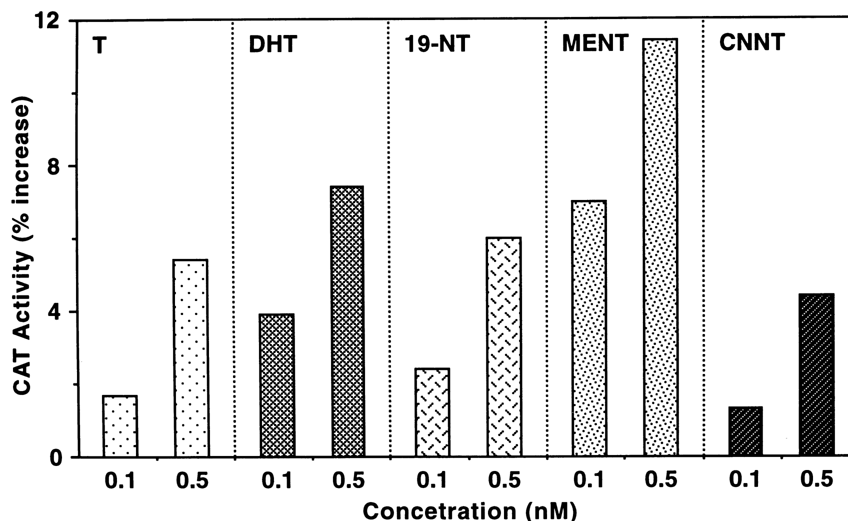

Fig. 7. Relative potency of androgens in a transient transfection assay in CV-1 cells transfected with a rat androgen receptor (AR) expression plasmid and a reporter plasmid encoding chloramphenicol acetyl transferase (CAT) under the control of mouse mammary tumor virus long terminal repeat (MMTV-LTR). Each dose of androgen was tested in triplicate. T, testosterone; DHT, dihydrotestosterone; CNNT, 7α-cyano-19-NT; 19-NT, 19-nortestosterone; MENT, 7α-methyl-19-NT.

lation of the CAT reporter (data not shown). The androgens, T, DHT, 19-NT, MENT and CNNT induced dose-dependent responses in CAT activity in the transfection assays (Fig. 7). MENT was found to be most active followed by DHT, 19-NT, T, and CNNT. The specificity of this response to androgens was established by blocking their activity using the antiandrogen, hydroxyflutamide (Fig. 8). The results of this in vitro study showed a good correlation between the binding affinity to AR and the activity in the transient transfection assays. The extent of activity in this in vitro assay is due to a direct effect of the compounds independent of metabolism and clearance. Hence, for 19-NT and DHT, whose androgenic potency in vivo was reduced significantly due to target organ specific metabolism or increased clearance, the activity in the transfection assays was not reduced and reflected more faithfully their binding affinity to AR.

4. Discussion

The present studies demonstrate that the mechanisms which determine the relative biopotency of T, 19-NT and its derivatives not only involve differences in their binding affinities to AR, but also their metabolism in the target tissues which leads to the formation

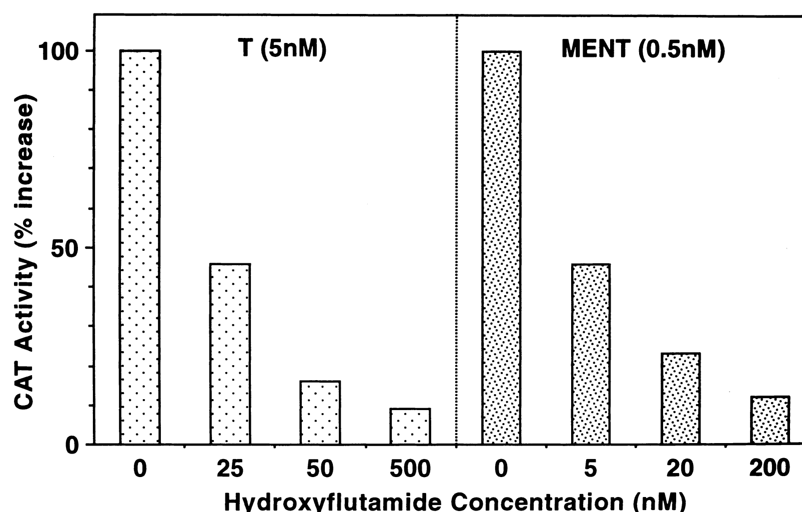

Fig. 8. Effect of hydroxyflutamide (an antiandrogen) on testosterone (T) and MENT stimulated CAT activity in the transfected CV-1 cells.

of compounds with greater or lesser activity than the parent compound.

It is well established that T is converted to DHT in the sex accessory glands, and since the binding affinity of DHT to AR is 3–5 times higher than that of T, its action is amplified in these tissues [8,10]. On the other hand, in castrated rats the potency of exogenously administered DHT was found to be either equal to or less than that of T. Similar reduction in the effect of exogenous DHT relative to T was observed in the maintenance of spermatogenesis in hypophysectomized rats [26]. However, DHT was shown to be 10 times more potent than T in in vitro transient transfection assays using CHO cells which are deficient in androgen metabolizing enzymes [27,28]. The decrease in androgenic activity of exogenously administered DHT could be due to its faster metabolism in rats. Since rats do not express SHBG, the metabolic clearance rate (MCR) of T and DHT is mainly dependent on liver and extrahepatic tissues. In the rat, the MCR of DHT (87 ± 9 L/100 g BW) is much higher than that of T (18 L/100 g BW) [29,30]. The difference in the MCR results in lower plasma levels of DHT compared to the levels of T when an equivalent amount of the two androgens are administered [31]. The reduction in the activity of exogenously administered DHT in rats cannot be extrapolated to other animals and man who possess SHBG in circulation. Exogenously administered DHT has been shown to be more potent than T in stimulating the growth of ventral prostate, seminal vesicles and bulbo-urethral glands in castrated Rhesus monkeys [32]. The overall metabolic clearance rate of DHT (764–630 L/day) is lower than that of T (1200–950 L/day) in normal men [33,34]. This is believed to be due to the higher affinity of DHT to SHBG compared to T [35], resulting in decreased availability of DHT for metabolism by liver enzymes [34].

For most steroids 5α-reduction in the liver is considered to be the rate limiting step in their metabolic clearance [36]. It was suggested that certain changes in the steroid structure makes them resistant to 5α-reduction in the liver, thereby decreasing their clearance and increasing their uptake by the target organ. Such a mechanism for the increased biopotency was proposed for synthetic glucocorticoids [37]. However, even though MENT cannot be 5α-reduced, it was cleared more rapidly than T in monkeys and men [12]. This is because MENT does not bind to SHBG [12], thus its clearance will not be hampered by binding to plasma proteins as is the case for T and DHT [34]. Testosterone is transported in plasma bound to SHBG, albumin, and in free form. Biologically active T represents both the albumin bound and free fraction since the half-dissociation time of T from albumin is rapid (<0.01 s), which is within the probable time of capillary transit through androgen target tissues [38,39]. The half-dissociation time of T from SHBG is relatively slow (>20 s), such that it is biologically not available at target tissues [40]. Since MENT does not bind to SHBG, its greater potency could also be due to a higher percentage of unbound MENT in the plasma in addition to its higher affinity to AR as compared to T. The higher concentration of unbound MENT may result in greater uptake by the target tissues, increasing its biopotency. A relationship between plasma protein binding and the relative biopotencies of substituted corticoids has been demonstrated earlier [41].

The biopotency differences between T and 19-NT in castrated rats, reflect yet another mechanism by which the activity of a steroid on the target-organ can be altered based on organ specific metabolism. Compared to T, 19-NT was found to be 5–6 times less potent on sex accessory glands but 3–4 times more potent as an anabolic agent. The differences in their androgenic vs anabolic potency are attributable to their 5α-reduction in the accessory glands but not in the muscle [17]. The binding affinity of 19-NT to AR is higher than that of T. But the opposite is the case for their 5α-reduced metabolites. In the prostate, 5α-reduction of 19-NT to DHNT lowers its binding affinity to AR, resulting in decreased androgenic activity in vivo [16]. In the case of MENT, the 7α-methylated derivative of 19-NT, its high potency could be attributed primarily to its greater affinity for AR and to its inability to undergo 5α-reduction.

It is clear from these studies that substitution at the 7α-position with a methyl group is important for enhanced binding affinity and increased biological activity since substitution with a cyano or a acetylthio moiety resulted in lower binding affinity and decreased biological activity. The importance of the α-configuration of the methyl group was further confirmed since a β-configuration led to a decrease in its androgenic potency. A similar effect of 7β-methyl configuration on the potency of DHT has also been reported [42]. This suggested that the conformational changes for increased receptor binding are specific for 7α-methylated derivatives. Liao and colleagues have proposed that the selectivity of androgen binding to its receptor is defined mainly by 7α- and/or 17α-position of the steroid structure. Substitution with a methyl group in these positions leads to a flattening of the steroid molecule which leads to a conformation that is less hindered for receptor binding [8,13]. Similarly, 7α-methyl substituent on estradiol has been shown to enhance its receptor binding affinity [43,44].

The correlation between biopotency and binding affinity was further evaluated in vitro by co-transfection of AR cDNA expression plasmid and a reporter plasmid encoding the CAT gene into CV-1 cells. In these assays MENT was found to be the most active

followed by DHT, 19-NT, T and CNNT. The specificity of this response to androgens was established by its inhibition with hydroxyflutamide, an antiandrogen. In these studies, there was a good correlation between binding affinity to AR and the in vitro activity in transfection assays. Thus, the androgenic potency of 19-NT and DHT in castrated rats did not correlate with either their receptor binding affinity or their CAT activity. The reason for the lower androgenic activity of the former is its metabolism to the less potent 5α-reduced derivative, and that of the latter is its faster clearance. Hence, factors other than receptor binding affinity have important physiological implications in the overall action of steroid hormones.

In conclusion, the biological activity of androgens is modulated by their binding to specific plasma proteins, their overall metabolic clearance, organ-specific metabolic transformation and binding affinity to androgen receptors. The increased relative anabolic activity of MENT is due to its higher affinity for androgen receptors and its resistance to 5α-reduction in the prostate.

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References

[1] J. Delettre, J.P. Mornon, G. Lepicard, T. Ojasoo, J.P. Raynaud, Steroid flexibility and receptor specificity, J. Steroid Biochem. 13 (1980) 45–59.

[2] J.P. Raynaud, T. Ojasoo, The relevance of structure-affinity relationships in the study of steroid hormone action, in: H. Eriksson, J.-A. Gustafsson (Eds.), Steroid Hormone Receptors: Structure and Function, Elsevier, Amsterdam, 1983, pp. 141–170.

[3] E. Dahlberg, A. Thalen, R. Brattsand, J.-A. Gustafsson, U. Johansson, K. Roempke, T. Saqrtok, Correlation between chemical structure, receptor binding, and biological activity of some novel, highly active, 16α,17α-acetal-substituted glucocorticoids, Molec. Pharmacol. 25 (1983) 70–78.

[4] K. Junkman, Long-acting steroids in reproduction, Recent Prog. Horm. Res. 13 (1957) 389–419.

[5] E. Nieschlag, H.M. Behre, Pharmacology and clinical uses of testosterone, in: E. Nieschlag, H.M. Behre (Eds.), Testosterone: Action, Deficiency, and Substitution, Springer–Verlag, Berlin, Heidelberg, 1990, pp. 92–114.

[6] A. Segaloff, The enhanced local androgenic activity of 19-nor steroids and stabilization of their structure by 7α- and 17α-methyl substituents to highly potent androgens by any route of administration, Steroids 1 (1963) 299–315.

[7] A. Segaloff, R.B. Gabbard, 14-dehydro-19-nortestosterone and its 7α-methyl derivative, Steroids 22 (1973) 99–105.

[8] S. Liao, T. Liang, S. Fang, E. Castaneda, T.-C. Shao, Steroid structure and androgenic activity, J. Biol. Chem. 248 (1973) 6154–6162.

[9] M.A. Avery, M. Tanabe, D.F. Crowe, G. Detre, R.H. Peters, W.K.M. Chong, Synthesis and testing of 17a, β- hydroxy-7α-methyl-D-homoestra-4,16-dien-3-one: a highly potent orally active androgen, Steroids 55 (1990) 59–64.

[10] N. Kumar, A.K. Didolkar, C. Monder, C.W. Bardin, K. Sundaram, The biological activity of 7α-methyl-19-nortestosterone is not amplified in male reproductive tract as is that of testosterone, Endocrinology 130 (1992) 3677–3683.

[11] K. Sundaram, N. Kumar, C.W. Bardin, 7α-methyl-nortestosterone MENT: The optimal androgen for male contraception, Ann. Med. 25 (1993) 199–205.

[12] N. Kumar, J. Suvisaari, Y.-Y. Tsong, C. Aguillaume, C.W. Bardin, P. Lähteenmki, K. Sundaram, Pharmacokinetics of 7α-methyl-19-nortestosterone in men and cynomologus monkeys, J. Androl. 18 (1997) 352–358.

[13] K.M.B. Chan, S. Smythe, S. Liao, Androgen receptor binding and androgenicity of methylated 4-ene-3-ketosteroids having no 17-hydroxy group, J. Steroid Biochem. 11 (1979) 1193–1196.

[14] S.A. Shain, R.W. Boesel, Saturation analysis of the binding of androgens, antiandrogens and estrogens by the cytoplasmic high affinity androgen receptor of the rat ventral prostate, J. Steroid Biochem. 6 (1975) 43–50.

[15] J. Steinsapir, M. Bryhan, T.G. Muldoon, Relative binding properties of microsomal and cytosolic androgen receptor species of the ventral prostate, Endocrinology 125 (1989) 2297–2311.

[16] F. Celotti, Cesi P. Negri, Anabolic steroids: a review of their effects on the muscle, of their possible mechanisms of action and of their use in athletics, J. Steroid Biochem. Molec. Biol. 43 (1992) 469–477.

[17] N. Kumar, K. Sundaram, C.W. Bardin, Feedback regulation of gonadotropins by androgens in rats: Is 5α-reductase involved?, J. Steroid. Biochem. Molec. Biol. 52 (1995) 105–112.

[18] V. Isomaa, A.E.I. Pajunen, C.W. Bardin, O.A. Jänne, Nuclear androgen receptors in the mouse kidney: Validation of a new assay, Endocrinology 111 (1982) 833–843.

[19] F.L. Graham, A.J. Eb Van der, A new technique for the assay of infectivity of human adenovirus 5 DNA, Virology 52 (1973) 456–467.

[20] J.J. Palvimo, P.J. Kallio, T. Ikonen, M. Mehto, O.A. Jänne, Dominant negative regulation of transactivation by the rat androgen receptor: roles of the N-terminal domain and heterodimer formation, Mol. Endocrinol. 7 (1993) 1399–1407.

[21] C.M. Gorman, L.F. Moffat, B.H. Howard, Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells, Molec. Cell. Biol. 2 (1982) 1044–1051.

[22] C.V. Hall, P.E. Jacob, G.M. Ringold, F. Lee, Expression and regulation of Escherichia coli lacZ gene fusions in mammalian cells, J. Molec. Appl. Genet. 2 (1983) 101–109.

[23] L.G. Hershberger, E.G. Shipley, R.K. Meyer, Myotrophic activity of 1-nortestosterone and other steroids determined by modified levator ani method, Proc. Soc. Exp. Biol. Med. 83 (1953) 175–180.

[24] N. Kumar, L.-X. Shan, M. Hardy, C.W. Bardin, K. Sundaram, Mechanism of androgen-induced thymolysis in rats, Endocrinology 136 (1995) 4887–4893.

[25] M.V. Govindan, Specific region in hormone binding domain is essential for hormone binding and transactivation by human androgen receptor, Mol. Endocrinol. 4 (1990) 417–427.

[26] M.E. Harris, A. Bartke, J. Weisz, D. Watson, Effects of testosterone and dihydrotestosterone on spermatogenesis, rete testis

fluid, and peripheral androgen levels in hypophysectomized rat, Fertil. Steril. 28 (1977) 1113–1137.
[27] J.-P. Deslypere, M. Young, J.D. Wilson, M.J. McPhaul, Testosterone and 5α-dihydrotestosterone interact differently with the androgen receptor to enhance transcription of the MMTV-CAT receptor gene, Mol. Cell. Endocrinol. 88 (1992) 15–22.
[28] Z.-X. Zhou, M.V. Lane, J.A. Kemppainen, F.S. French, E.M. Wilson, Specificity of ligand-dependent androgen receptor stabilization: Receptor domain interactions influence ligand dissociation and receptor stability, Mol. Endocrinol. 9 (1995) 208–218.
[29] D.K. Lee, C.E. Bird, A.F. Clark, In vivo metabolism of 3H-testosterone in adult male rats: effects of estrogen administration, Steroids 26 (1975) 137–147.
[30] E.J. Van Doorn, B. Burns, D. Wood, C.E. Bird, A.F. Clark, In vivo metabolism of ^{3}H-dihydrotestosterone and ^{3}H-androstanediol in adult male rats, J. Steroid Biochem. 6 (1975) 1549–1554.
[31] W. Bartsch, C. Knabbe, V. Klaus-Dieter, Regulation and compartmentalization of androgens in rat prostate and muscle, J. Steroid Biochem. 19 (1983) 929–937.
[32] N. Dinakar, R. Arora, M.R.N. Prasad, Effects of microquantities of 5α-dihydrotestosterone on the epididymis and accessory glands of the castrated Rhesus monkey, Macacca mulatta, Int. J. Fertil. 19 (1974) 133–139.
[33] R. Horton, J. Shinsako, P.M. Forsham, Testosterone production and metabolic clearance rates with volume of distribution in normal adult men and women, Acta Endocrinol. (Copenhagen) 48 (1965) 446–458.
[34] T. Ishimaru, W.A. Edmiston, L. Pages, R. Norton, Splanchnic extraction and conversion of testosterone and dihydrotestosterone in man, J. Clin. Endocrinol. Metab. 46 (1978) 528–533.
[35] W. Heyns, P. De Moor, Kinetics of dissociation of 17β-hydroxysteroids from the steroid binding β-globulin of human plasma, J. Clin. Endocrinol. Metab. 32 (1971) 147–154.
[36] A.F. Clark, Steroid Δ^4-reductases: their physiological role and significance, in: R. Hobkirk (Ed.), Steroid Biochemistry, Vol. 1, CRC Press, Florida, 1979, pp. 1–27.
[37] J.R. Florini, L.L. Smith, D.A. Buyske, Metabolic fate of a synthetic corticosteroid (triamcinolone) in the dog, J. Biol. Chem. 236 (1961) 1038–1042.
[38] W.M. Pardridge, Transport of protein bound hormone into tissues in vivo, Endocrine Rev. 2 (1981) 103–123.
[39] W.M. Pardridge, Serum bioavailability of sex steroid hormones, Clin. Endocrinol. Metab. 15 (1986) 259–278.
[40] D.A. Damassa, A.W. Gustafson, Effect of chronic infusions of sex steroid-binding protein on the testosterone-mediated inhibition of gonadotropin secretion and maintenance of sex accessory glands in male rats, Endocrinology 123 (1988) 1885–1892.
[41] J.R. Florini, D.A. Buyske, Plasma protein binding of triamcinolone-H3 and hydrocortisone-4-C14, J. Biol. Chem. 236 (1961) 247.
[42] W.H. Chin, M.E. Wolff, Synthesis and biological activities of 7β-methyl steroids, J. Med. Chem. 22 (1979) 1257–1260.
[43] F.J. Zeelen, E.W. Bergink, Structure-activity relationships of steroid estrogens, in: J. Raus, H. Martens, G. Leclercq (Eds.), Cytotoxic Estrogens in Hormone Receptive Tumours, Academic Press, London, 1980, pp. 39–48.
[44] A. LaMorte, N. Kumar, C.W. Bardin, K. Sundaram, Aromatization of 7α-methyl-19-nortestosterone by human placental microsomes in vitro, J. Steroid Biochem. Molec. Biol. 48 (1994) 297–303.