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EFFECTS OF ANTIANDROGENS ON GROWTH OF ANDROGEN-DEPENDENT MOUSE MAMMARY TUMOR (SHIONOGI CARCINOMA 115) *IN VIVO* AND *IN VITRO**

T. SUZUKI,† I. HORIBE, N. UCHIDA, K. EZUMI, K. UCHIDA, K. TAKEDA, A. TANAKA,[1] Y. NISHIZAWA[1] and K. MATSUMOTO[1]

Shionogi Research Laboratories, Fukushima-ku, Osaka 553 and [1]Department of Pathology, Osaka University Medical School, Kita-ku, Osaka 530, Japan

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Summary—Binding affinities of modified steroidal anthrasteroids, 3β-hydroxy-3aβ,6-dimethyl-2,3,3a,4,5,8,9,10,10aβ,11,11aβ,11bα-dodecahydro-1H-cyclopenta[*a*]anthracene-8-one (**1**) and 3aβ,6-dimethyl-2,3,3a,4,5,8,9,10,10aβ,11,11aβ,11bα-dodecahydro-1H-cyclopenta[*a*]anthracene-3,8-dione (**2**), the steroid oxendolone and the nonsteroid AA560, for the androgen receptor (AR) of Shionogi carcinoma 115 (SC115) and their effects on the growth of SC115 were investigated *in vivo* and *in vitro*. The inhibitory effects of these compounds on testosterone 5α-reductase of SC115 tissues were also measured. The relative binding affinities of these compounds were 3.17–0.03% of that of dihydrotestosterone, and their rank order was (**1**) > AA560 > oxendolone ≫ (**2**).

In the presence of 10^{-9} M testosterone, anthrasteroids and AA560 inhibited the growth of SC115 cells at 10^{-7} M in a serum-free medium, but oxendolone did not. In the absence of testosterone, (**1**), (**2**) and oxendolone promoted cell growth at 10^{-6}, 10^{-7} and 10^{-7} M, respectively. However, AA560 nearly completely blocked cell growth at 10^{-5} M.

At a 2 mg daily dose for 13 days, (**1**) and AA560 powerfully inhibited tumor growth in castrated DS mice treated with testosterone propionate but oxendolone had almost no effect. Anthrasteroids and oxendolone showed weak but significant agonistic activity *in vivo*. Anthrasteroids markedly inhibited 5α-reductase activity of SC115, oxendolone weakly and AA560 not at all. The remarkable antiandrogenic activities of (**1**) and AA560 may partially result from their higher affinities for the AR of SC115 but other yet unknown mechanisms may also contribute to these activities.

INTRODUCTION

There are many steroidal antiandrogens which have complex profiles of action that involve mechanisms other than androgen receptor (AR) antagonism. For example, cyproterone acetate not only competes with androgen for AR in target organs but also inhibits gonadotropin secretion [1], and oxendolone (19-nortestosterone derivative) is reported to be an AR antagonist and an inhibitor of 5α-reductase, the enzyme that catalyzes the conversion of testosterone to 5α-dihydrotestosterone (DHT), in the prostate [2]. The contribution of each mechanism to the action of such steroid derivatives will change depending on experimental animals, target tissues and duration of treatment.

Recently, we were interested in how modification of the androstane skeleton can bring about the decrease or loss of androgenic properties and acquirement of antiandrogenic properties and found that anthrasteroid **1** had strong antiandrogenic activity in castrated rats treated with testosterone propionate (TP) [3]. In order to characterize the action of anthrasteroids, we examined their activities on the growth of androgen-dependent mouse mammary Shionogi carcinoma 115 (SC115) [4] *in vivo* and in a cell culture system. As SC115 cells grow rapidly and androgen-dependently, they are a particularly appropriate model to determine agonistic and antagonistic activities of a test compound effectively [5]. Since the SC115 tumor has low 5α-reductase activity [6, 7], we measured inhibition of 5α-reductase activity by the test compounds. As reference compounds we chose the steroid oxendolone and nonsteroid AA560 which is a trichloraniline derivative [8].

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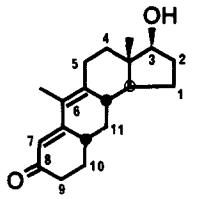
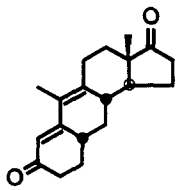
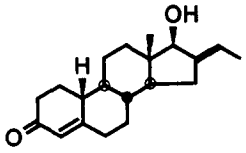
†To whom correspondence should be addressed.



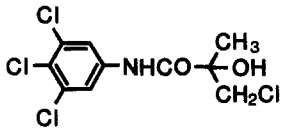
Anthrasteroid 1



Anthrasteroid 2



Oxendolone



AA560

Scheme 1. Structural formula.

EXPERIMENTAL

Chemicals

[^{3}H]Methyltrienolone ([^{3}H]MT, 3.22 and 3.03 TBq/mmol) and [1α,2α-^{3}H]testosterone ([^{3}H]T, 1.48 TBq/mmol) were obtained from duPont–New England Nuclear and purified by silica gel TLC (chloroform:methylalcohol, 39.2:0.8, by vol) before use. Anthrasteroid **1** (3β-hydroxy-3aβ,6-dimethyl-2,3,3a,4,5,8,9,10,10aβ,11,11aβ,11bα-dodecahydro-1H-cyclopenta[*a*]-anthracene-8-one), **2** (3aβ,6-dimethyl-2,3,3a,4,5,8,9,10,10aβ,11,11aβ,11bα-dodecahydro-1H-cyclopenta[*a*]anthracene-3,8-dione) and AA560 (*N*-(2-chloromethyl-2-hydroxy-propionyl)3,4,5-trichloroaniline) were synthesized by us [9]. Oxendolone (16β-ethyl-17β-hydroxy-4-oestren-3-one) was purchased from Takeda Chemical Industries Ltd and was recrystallized from diethylether–dichlormethane. The other chemicals were of analytical grade.

Buffer solutions and preparation of cytosol

The method was similar to that described previously [10]. Briefly, phosphate buffer (P buffer: pH 7.5, 0.02 M KH_2PO_4-Na_2HPO_4 containing 0.5 mM EDTA-Na_2, 10 mM Na_2MoO_4, 0.5 mM dithiothreitol and 0.25 M sucrose) was used for the homogenization of the tumors. Test compounds dissolved in ethanol were diluted with P′ buffer (P buffer without dithiothreitol and sucrose containing 10% (v/v) of ethylene glycol). Final concentrations of ethanol and ethylene glycol in the binding reaction were less than 1 and 2.5%, respectively. Fragments of SC115 were grafted on DS mice (5 weeks old) from our Aburahi Laboratories (Shiga). About 4 weeks after the implantation, the animals were sacrificed and tumor tissues were removed and stored at −80°C immediately. The tumors were thawed and homogenized in 4–7 volumes of P buffer by a glass–glass homogenizer at 0°C. The homogenate was centrifuged for 2 h at 200,000 *g* (0°C, Beckman L8-70M, Ti60 roter) to obtain the supernatant (cytosol). The cytosol was used for estimating binding to AR.

AR binding

Displacement curves. A 100 μl aliquot of the cytosol was incubated with 50 μl of a constant concentration of [^{3}H]MT (4–7 × 10^{-9} M) and 50 μl of various concentrations (4 × 10^{-10}–1 × 10^{-5} M) of the test compounds or P′ buffer for the control tube in small glass tubes.

Scatchard plot analysis. A 100 μl aliquot of the cytosol was incubated with 50 μl of various concentrations (0.2 to 32 × 10^{-9} M) of [^{3}H]MT and 50 μl of the constant concentration of the test compounds (4 × 10^{-9}–4 × 10^{-6} M) or P′ buffer for the control tube. After incubation of the tubes for 20 h at 0°C, 200 μl of dextran-coated charcoal (1.0% Norit A, 0.10% dextran T70, P′ suspension) was added to separate bound from free steroid. The tubes were incubated for 10 min, were then centrifuged for 15 min at 3000 rpm, and a 200 μl of the supernatant was counted in a liquid scintillation counter (Aloka LSC-1000) in 5 ml of Aquasol-2 (New England Nuclear) with a 48–49% counting efficiency.

Nonspecific binding was determined in parallel incubations with a 100-fold excess of unlabeled MT or DHT in the tube containing the

highest concentration of [^{3}H]MT. Nonspecific binding of the other tubes was calculated from this value in proportion to the concentration of [^{3}H]MT added. Specific binding was obtained by subtracting nonspecific binding from total binding. Relative binding affinity (RBA) was calculated as follows:

$$\text{RBA} = 100 \times {}^{50}_{0}\text{C}/{}^{50}\text{C}\,(\%), \quad \text{DHT} = 100\,(\%)$$

where ${}^{50}_{0}$C and ^{50}C were respectively the concentrations of DHT and test compounds required for 50% inhibition of control binding.

Association contants (K_a) of MT and the test compound (K_s) for AR were obtained from Scatchard plot analysis [11].

Cell culture

The methods for primary culture of SC115 cells and cloning of the cells by the limiting dilution method have been reported previously [12]. One of the androgen-dependent cell lines obtained was used in this study. SC115 cells were plated at 5×10^3 cells/well into a 96-well plate (Costar, Cambridge, Mass) containing 0.15 ml MEM added with 2% dextran-coated charcoal-treated fetal calf serum. On the following day, the medium was changed to 0.15 ml serum-free medium [Ham's F-12:MEM (1:1, v/v) containing 0.1% BSA] in the presence or absence of 10^{-9} or 10^{-8} M of testosterone with or without various concentrations (10^{-10}–10^{-5}) of the test compounds, and the cells were cultured for 4 days at 37°C. On the fourth day, [^{3}H]thymidine (5.9 kBq/well) was added to each well and incubated further for an hour. The radioactivity incorporated into the cells was counted as described previously [13]. When the effects of various concentrations of testosterone on both number of SC115 cells on day 6 and [^{3}H]thymidine uptake on day 4 were examined in the serum-free culture [13], parallel increases were observed. However, the growth stimulation estimated by [^{3}H]thymidine was slightly higher than that estimated by cell number [13]. DHT added in the serum-free medium was slightly more effective than testosterone in growth stimulation of SC115 cells at lower concentrations (10^{-10}–10^{-9} M) but was similar to testosterone at higher concentrations (10^{-8}–10^{-6} M) [12].

In vivo experiments

Antagonistic activity. Male DS mice (5–6 weeks old, 7 animals per group) were used. 4 or 5 days after castration, the backs of the mice were grafted subcutaneously with 2–3 mm^3 of tumor fragment. TP (0.1 mg/mouse/day) was administered subcutaneously from the day of implantation throughout the experiment. The test compounds dissolved or suspended in the vehicle (saline, 0.4% Polysorbate 80, 0.5% carboxymethyl cellulose, and 0.9% benzyl alcohol) or the vehicle alone (control group) were given subcutaneously or by the oral route once daily for 13 or 20 days. On the day following the last administration, the animals were autopsied and wet weights of the tumor and prostates were measured. The inhibitory activity of the compounds was expressed as the ratio of the tumor or prostate weights of the treated and control groups.

Agonistic activity. Male DS mice (6 weeks old, 7 animals per group) were castrated 6 days before inoculation. Test compounds (1 and 4 mg/mouse/day) were administered subcutaneously for 21 days. DHT (0.05 mg/mouse/day) was given for only 13 days. The mice were dissected on day 22 (day 14 for the DHT group) and the weights of the tumor and prostates were determined.

5α-Reductase inhibition

The preparation of nuclear membrane fraction of the SC115 tissues and measurements of 5α-reductase activity were performed according to the method of Moore and Wilson [14] with minor modifications. The reaction mixtures of 0.5 mM NADPH, the enzyme suspension, and various concentrations (0.69–9.6×10^{-8} M) of [^{3}H]T in the presence and absence of test compounds (0.31 or 3.1×10^{-7} M) were incubated for 1 h at 25°C. Steroids in reaction mixtures were extracted with chloroform–methanol (2:1, v/v). Aliquots of the extracts were evaporated by nitrogen gas and the residues were dissolved in small amounts of methanol containing unlabeled androstanediol (A-diol), DHT and testosterone as carriers. The steroids produced were separated by TLC (Whatman, PE SIL G; solvent system: chloroform–methanol, 39:1, v/v) and the radioactivities of each zone corresponding to A-diol, DHT and testosterone were counted. Values of K_m and inhibition constant of the test compounds (K_i) were obtained by a Lineweaver–Burk plot [15]. Protein concentrations of the enzyme suspension were determined by the method of Lowry *et al.* [16].

Superposition method of test compounds

Estimation of the three dimensional structures of anthrasteroid **1**, DHT and AA560 was carried out by using the molecular modeling program SYBYL [17].

Comparison of the overall shapes of anthrasteroid **1** and DHT was carried out by overlapping of the least-squares planes for the A- and B-rings, and of the O–O lines projected on to these planes. Furthermore, to compare the structure of AA560 with those of anthrasteroid **1** and DHT, a phenyl ring of AA560 was superposed to the least-squares planes of **1** and DHT, whilst adjusting to the margins of the van der Waals surfaces of the para chlorine atom of AA560 and the A-ring oxygen atoms of **1** and DHT. The hydroxyl oxygen of AA560 was fitted to the D-ring oxygens of **1** and DHT as closely as possible.

RESULTS

Androgen receptor binding

Figure 1 shows displacement curves. The RBA of anthrasteroid **1** for AR of SC115 was 3.2% of that of DHT. Anthrasteroid **2**, which is the 17-keto-derivative of **1**, had very low affinity, about one hundredth of that of anthrasteroid **1** (Table 1).

Figure 2 shows typical Scatchard plots of [^{3}H]MT binding to AR in the presence and absence of the test compounds. From the intercepts at the ordinate of the straight lines for the control and the test compounds, respectively, the $K_a(R_0)$ and $K_a(R_0)/(1 + K_s(I))$ values were calculated. The slope of the control line gave the association constant (K_a) of MT for AR. The association constants (K_s) of the test compounds for AR were calculated from the K_a value and the concentration of the test compound (I) [11]. The ratios of association constants of the test compounds compared to that of DHT are given in Table 2. The order of the affinities is

anthrasteroid **1** > AA560 > oxendolone

≫anthrasteroid **2**.

These results from Scatchard analysis were comparable with the RBA values shown in Table 1.

Table 1. Relative binding affinities (RBA) of the test compounds for AR of SC115

Compound	No. of experiments	RBA[a] (%, DHT = 100)
Anthrasteroid **1**	6	3.17 ± 0.18
Anthrasteroid **2**	5	0.033 ± 0.004[b]
Oxendolone	5	0.94 ± 0.17
AA560	5	2.28 ± 0.09
Testosterone	8	18.7 ± 1.2

The cytosol of SC115 was incubated with various concentrations (0.1–10,000 nM) of a test compound and constant concentration (0.97–1.7 nM) of [^{3}H]MT for 20 h at 0°C. Bound and free steroids were separated by the dextran-coated charcoal method. RBA was obtained from the displacement curves as described in Experimental. [a]Mean ± SE. [b]Extrapolated value.

Effects on the growth of SC115 cells in culture

In the presence of 10^{-9} M testosterone alone, [^{3}H]thymidine uptake of the cells increased over 10-fold. Therefore, testosterone was an effective promoter of DNA synthesis (Fig. 3a and b). In the presence of 10^{-9} M testosterone,

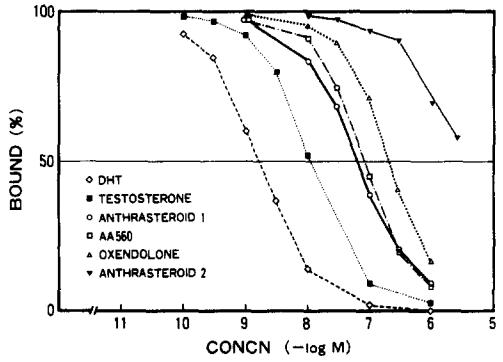


Fig. 1. Displacement curves of the [^{3}H]MT binding to AR of SC115 with test compounds and androgens. The cytosol was incubated with 0.97 nM of [^{3}H]MT and various concentrations of anthrasteroids **1** and **2**, oxendolone, AA560, testosterone or DHT for 20 h at 0°C. Bound and free steroids were separated by the dextran-coated charcoal method. RBA of the test compounds for AR were determined as described in Experimental.

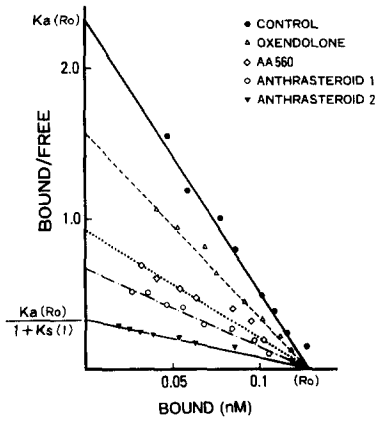


Fig. 2. Scatchard plots of [^{3}H]MT binding to AR of SC115 in the presence and absence of test compounds. The cytosol of SC115 tumors was incubated with various concentrations of [^{3}H]MT in the presence and absence of 10 nM of the anthrasteroid **1**, oxendolone and AA560 or 1000 nM of anthrasteroid **2** for 20 h at 0°C. Bound and free steroids were separated by the dextran-coated charcoal method. Association constants (K_a, K_s) of [^{3}H]MT and test compounds were calculated as described in Experimental.

Table 2. Association constants of the test compounds for AR of SC115

Compound	No. of experiments	Concentration (nM)	K_s, K_a[a] (M^{-1})	Ratio[b]
Anthrasteroid **1**	6	10	$2.7 \pm 0.57 \times 10^8$	2.5
Anthrasteroid **2**	5	1000	$9.1 \pm 0.89 \times 10^6$	0.08
Oxendolone	7	10	$0.88 \pm 0.17 \times 10^8$	0.80
AA560	7	10	$1.8 \pm 0.32 \times 10^8$	1.6
Testosterone	7	1	$1.8 \pm 0.36 \times 10^9$	16
DHT	4	1	$1.1 \pm 0.13 \times 10^{10}$	100
[^{3}H]MT	7	—	$1.8 \pm 0.26 \times 10^{10}$	160

The cytosol of SC115 was incubated with a constant concentration (1–1000 nM) of the test compound and various concentrations of [^{3}H]MT (0.05–7.9 nM) for 20 h at 0°C. Bound and free steroids were separated by the dextran-coated charcoal method. Association constants of the test compound (K_s) and [^{3}H]MT (K_a) for AR were obtained by Scatchard plots described in the Experimental.

[a] Mean ± SE.

[b] Ratio of association constants ($K_{rDHT} = 100$).

10^{-8}–10^{-7} M anthrasteroid **1** decreased the uptake of radioactivity by the cells in a concentration-dependent manner. At 10^{-6} M the inhibition became less than at 10^{-7} M, and at 10^{-5} M there was stimulation of uptake. Anthrasteroid **2** shows a similar curve to anthrasteroid **1**, but maximum inhibition occurred at 10^{-6} M. Oxendolone failed to inhibit the androgen-induced growth of the cells over all tested concentrations. AA560 inhibited this growth from 10^{-8} M and completely suppressed it at 10^{-5} M.

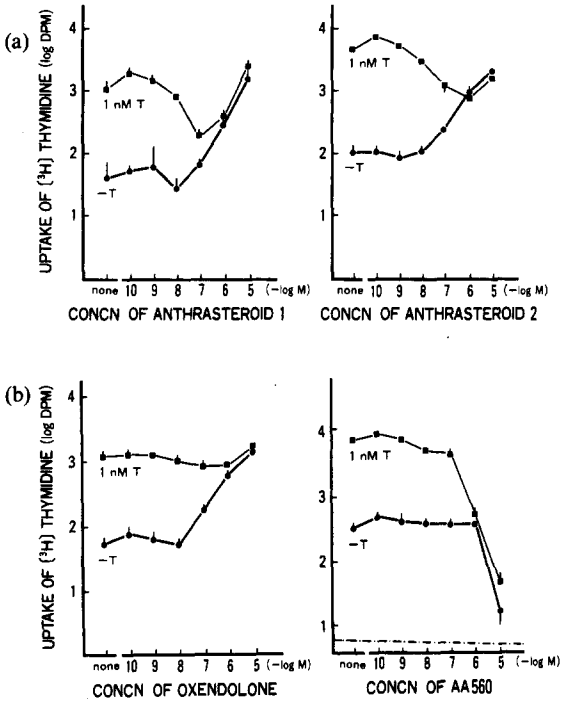


Fig. 3. Effects of anthrasteroids **1** and **2**, oxendolone and AA560 on the growth of SC115 cells in serum-free culture. SC115 cells (5000 cells/well) were cultured in the medium containing 0.1 nM–10 μM of antiandrogens in the presence or absence of 1 nM of testosterone for 4 days at 37°C. On the fourth day, [^{3}H]thymidine (5.9 kBq/well) was added and the cells were incubated for an additional hour. The uptake of [^{3}H]thymidine by the cells was counted.

In the presence of 10^{-8} M testosterone, the inhibition curves of these compounds were almost parallel to those in the presence of 10^{-9} M testosterone (data not shown).

In the absence of testosterone, anthrasteroids **1** and **2** increased [^{3}H]thymidine uptake in the concentration range of 10^{-7}–10^{-5} M. Oxendolone stimulated the cell growth more clearly at 10^{-7}–10^{-5} M in a concentration-dependent manner. In contrast, AA560 alone showed no stimulatory effects at 10^{-6} M but blocked the uptake of radioactivity completely at 10^{-5} M.

Effects on the growth of SC115 tumors in vivo

Anthrasteroid **1** and oxendolone were given subcutaneously. AA560 was administered by the oral route in a dosage of 0.1–2 mg. Both anthrasteroid **1** and AA560 markedly inhibited tumor growth in castrated mice treated with TP, whereas oxendolone had almost no effect (Fig. 4a). Anthrasteroid **1** also exhibited antitumor activity on oral administration at a daily dose of 0.2–5 mg for 20 days in intact mice, whereas its 17-keto-derivative **2** showed weaker inhibition. At the highest dosage of 5 mg, it inhibited growth by 50% (Fig. 4b). Anthrasteroid **1** and AA560 also markedly suppressed the androgen-induced weight increase of the prostates of the tumor-bearing mice. For this tissue, oxendolone revealed a very weak inhibition (Fig. 5). The agonistic activity of anthrasteroids or oxendolone was measured by administration of the test compounds alone for 22 days. Anthrasteroids, **1**, **2** and oxendolone had weak but significant agonistic activity. Oxendolone showed more agonistic activity than antrasteroids at a 4 mg dose. DHT administered for 14 days increased tumor weight by up to 120-fold over control (Fig. 6). These results of agonistic activity were comparable with those of cell culture experiments. Anthrasteroids **1**, **2**

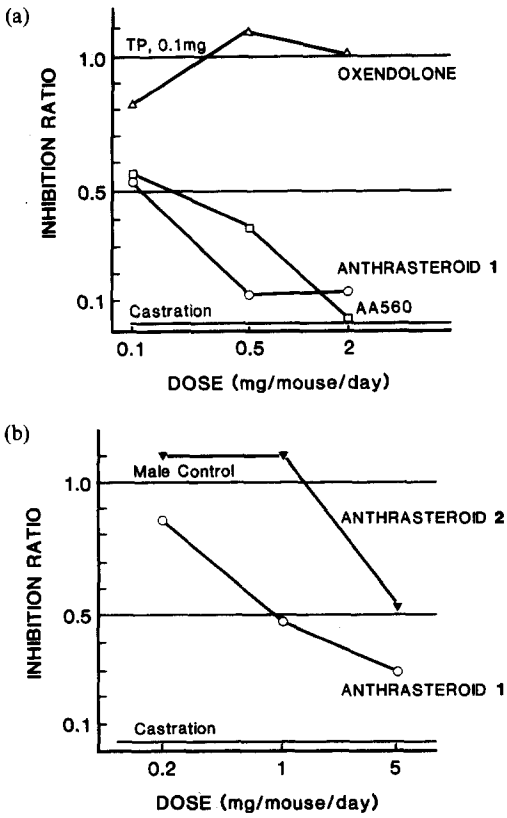


Fig. 4. (a) Inhibition of androgen-induced growth of SC115 tumor by anthrasteroid **1**, oxendolone and AA560 *in vivo*. Male DS mice (6 weeks old) were castrated and grafted with SC115 tumor fragments. TP (0.1 mg/day/mouse) was injected subcutaneously during the experiment. Anthrasteroid **1** and oxendolone were injected subcutaneously, and AA560 was administered by the oral route for 13 days. The weights of the tumor were measured on the day following the last treatment. (b) Inhibition of the growth of SC115 tumor by anthrasteroids **1** and **2**, in intact mice. Intact male DS mice were grafted with SC115 tumor fragments. The mice were administered with a 0.2–5 mg daily doses of anthrasteroids **1** and **2**, for 20 days by the oral route. The weights of the tumor were measured on the day following the last administration.

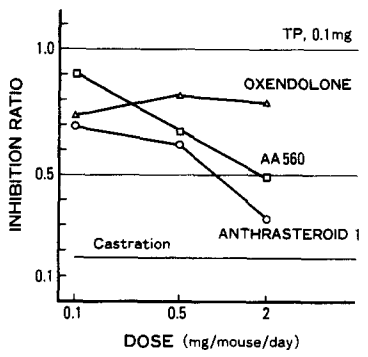


Fig. 5. Inhibition of androgen-induced increases in the weights of the prostate of tumor-bearing DS mice by anthrasteroid **1**, oxendolone and AA560. Male DS mice (6 weeks old) were castrated and grafted with SC115 tumor fragments. The mice were injected subcutaneously with TP (0.1 mg/day/mouse) during the experiment. Anthrasteroid **1** (○) and oxendolone (△) were injected subcutaneously, and AA560 (□) was administered orally for 13 days. The weights of the prostate were measured on the day following the last treatment.

and oxendolone also increased the prostatic weights of the tumor-bearing mice, respectively by 1.5-, 1.4- and 1.5-fold over control at a 1 mg dose.

5α-Reductase inhibition

The inhibitions by both anthrasteroids and oxendolone were found to be competitive and the conversion percent of testosterone [14] in the presence and absence of the enzyme were 1.7–2.4% and 0.6–0.9%, respectively. The values of K_m and K_i are given in Table 3. Anthrasteroids **1** and **2** showed high inhibitory activity. The inhibitory activity of oxendolone was lower than that of the anthrasteroids but AA560 had no inhibitory activity at the concentration of 3.1×10^{-7} M. The specific activity [14] of the nuclear membrane fraction of SC115 was 2.4 ± 0.1 unit per mg protein at 5.5–6.5 nM of [^{3}H]T. In a few experiments with rat prostatic nuclear fraction, we have obtained similar inhibitory activities of these compounds for 5α-reductase.

DISCUSSION

The current theory for the mechanism of steroid hormone action is: (1) hormone binds to the receptor in the cell of the target organ [18]; (2) hormone binding alters receptor structure

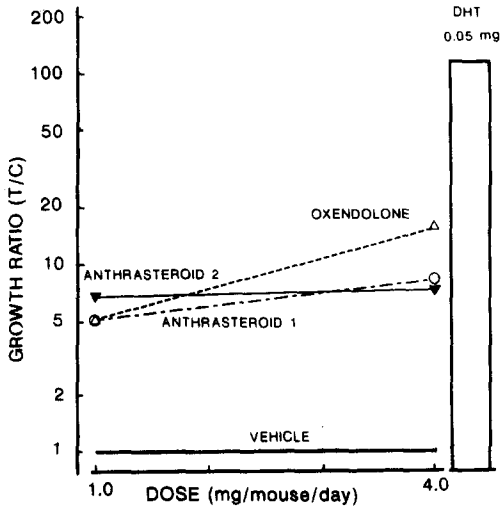


Fig. 6. Agonistic activity of anthrasteroids and oxendolone. Castrated male DS mice were subcutaneously inoculated with SC115 fragment on day 0. Test compounds and DHT were subcutaneously administered for 21 and 13 days, respectively. Tumor weight of test compound group was expressed as ratio to the weight of vehicle control group.

Table 3. Inhibition of 5α-reductase of SC115

Compound	No. of experiments	Inhibition constant ($\times 10^{-8}$ M)
Anthrasteroid 1	2	3.1, 5.4
Anthrasteroid 2	1	4.7
Oxendolone	2	10, 10
AA560	1	—
Progesterone	1	2.2
None	4	15 ± 2^a

The assay mixtures were incubated for 1 h at 25°C using [^{3}H]T as substrate [14]. The inhibition constants were obtained from Lineweaver–Burk plots [15].
[a] K_m Value (mean ± SE).

and the activated hormone–receptor complexes thus formed bind to the hormone responsive element (HRE) of DNA with high affinity and enhance transcription [19, 20].

The reaction mechanisms of antiandrogens in the above AR binding series might consist of their competitive binding to AR, prevention of AR conformational change required for hormonal expression [21] and masking binding of AR–androgen complex to DNA [22] by AR–antiandrogen complexes.

The RBAs of anthrasteroid **1**, AA560 and oxendolone for AR of SC115 were moderate, corresponding to about 16, 10 and 5% of that of testosterone, respectively. It has already been reported that oxendolone has moderate affinity ($K_d = 5.0 \times 10^{-9}$ M) [24], whereas AA560 has very low affinity for rat prostatic AR [8]. Anthrasteroid **1** also showed moderate affinity for AR of rat prostate (RBA: about 1.3% of DHT, unpublished data). The reasons for the discrepancy in the affinities of these compounds for AR of SC115 and rat prostates are not clear. The discrepancy could be due to differences in associated substances in crude AR preparations or the metabolism of the compounds involved in the binding reaction.

Anthrasteroid **2** showed very low RBA compared with **1**, since 17-ketone substitutions of 17β-hydroxyl of androstane derivatives decreased AR affinity dramatically [23].

Table 4. O–O Distance of test compounds

Compound	O–O Distance (Å) Observed	Calculated[a]
Anthrasteroid 1	11.05[b]	11.31
Anthrasteroid 2		11.05
Oxendolone		10.92
AA560		8.99*
Testosterone	10.94[c]	10.97
DHT	10.77[d]	11.02

[a] Distances were estimated for the structures calculated by Maximin II in SYBYL.
[b] Private communication from Dr M. Shiro.
[c] Ref. [27].
[d] Ref. [28].
*p-Cl–O distance.

It has been indicated that in AR binding, the bulkiness and flatness of the steroid molecules play a more important role than the electronic effects at the Δ^4 bond of the A-ring [25]. It has been emphasized that in AR binding the functional groups at the A- and D-ring ends of the steroid are significant [21]. Figure 7 shows that the conformation of anthrasteroid **1** approximately overlaps that of DHT with regard to the A-, B- and C-rings. The carbonyl oxygen of the A-ring of anthrasteroid **1** is nearer to the least square plane than that of DHT, but the hydroxyl oxygen of the D-ring of **1** is farther below the plane than that of DHT. These differences might cause the decrease in affinity for AR and also antagonistic activity. For the oxendolone molecule, Duax *et al.* have pointed out that the ethyl substituent at C(16) interferes with receptor interaction either directly or by inhibiting a required interaction with O(17) [21]. p-Chlorine and hydroxyl groups of AA560 fit well in the plane. The chlorine is apart from the carbonyl oxygen of DHT though the hydroxyl is close to that of DHT.

Anthrasteroids and oxendolone both showed weak but significant agonistic activity. Due to insufficient stock, we could not test the effects of AA560 alone *in vivo*. However, the compound was devoid of agonistic activity in cell culture. Oxendolone has been reported to give a slight androgenic response on rat ventral prostate [2]. Anthrasteroid **1** did not increase the weight of prostates of immature castrated rats at a daily dose of 1 mg per animal for 7 days, but it revealed androgenic activity in castrated mature rats at a 30 mg/kg dose for 10 days (unpublished data).

In an interaction of the AR–androgen complex with DNA, if two stereospecific hydrogen bonds to DNA were formed via the carbonyl group and hydroxyl group of the A- and D-rings of androgen [22], the distance between the hydroxyl and keto oxygen atoms might be critical for development of an agonist response. Anthrasteroids and oxendolone have O–O distances very similar to those of testosterone and DHT. This similarity might account for their weak agonistic activity although this hypothesis would have to be examined for many compounds. The fact that AA560 has no agonist activity may be correlated with its short *p*-Cl–O distance.

With regard to the antiandrogenic activity of the compounds, anthrasteroid **1** and AA560 strongly inhibited the androgen-induced growth

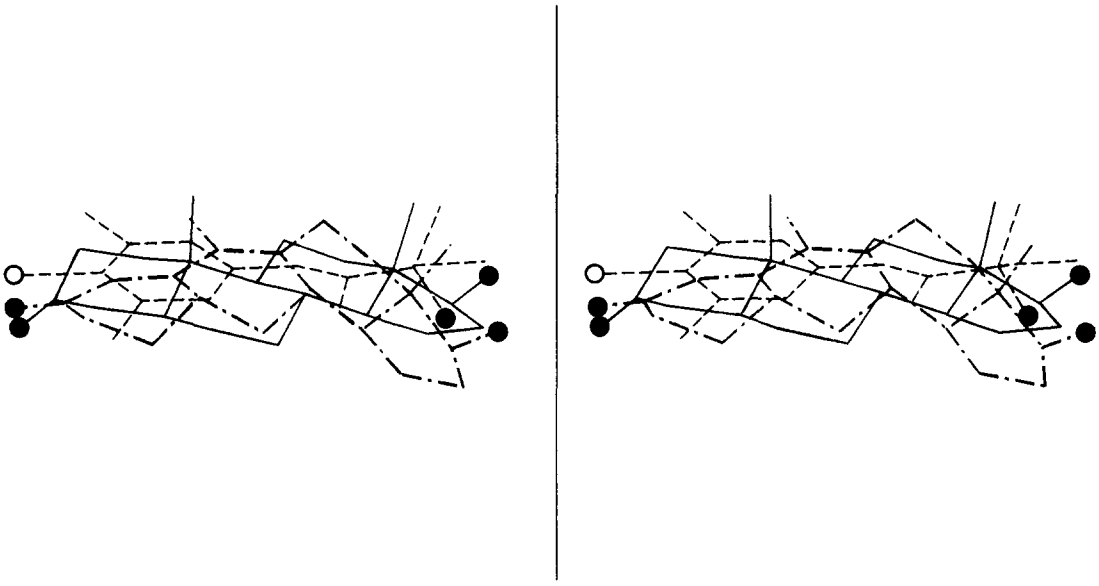


Fig. 7. A stereo view comparison of overall conformations of anthrasteroid **1** (—·—) and AA560 (-·-·-) with that of DHT (——). ●; oxygen, ○; chlorine atom.

of SC115 in cell culture as well as *in vivo*, whereas oxendolone was almost ineffective in both cases. Although anthrasteroid **2** had a very low RBA, it suppressed androgen-induced growth of tumor cells in culture. This discrepancy can be explained by our findings that anthrasteroid **2** is very rapidly metabolized to anthrasteroid **1** under the culture conditions (unpublished data). *In vivo*, anthrasteroid **2** showed a weaker antagonistic effect than anthrasteroid **1**.

5α-Reductase activity in SC115 tumor is lower than that in androgen-dependent tissues such as seminal vesicles and ventral prostates but is higher than that in androgen-independent tissues such as liver and muscle [7]. Bruchovsky reported that DHT was more active than T in SC115 tumor growth and that a small amount of DHT was recovered from SC115 tumors when [^{3}H]T was administered to tumor-bearing mice [6]. The obtained K_m value of 5α-reductase of SC115 was larger than that of rat prostatic nuclear fraction ($K_m = 2.5 \times 10^{-8}$ M), and the specific activity of the enzyme in SC115 was lower than that in rat prostate (2.5 unit/mg protein, both unpublished data).

Anthrasteroids had higher 5α-reductase inhibitory activity than oxendolone which has been reported to inhibit 5α-reductase activity in rat prostate [26]. AA560 did not show any inhibitory activity. Anthrasteroids might at least partially retard the growth of SC115 and ventral prostate by inhibiting the 5α-reductase activities of these tissues. Although oxendolone inhibited the growth of ventral prostate of tumor-bearing mice, it did not show inhibitory effect on tumor growth. This phenomenon may be due to its weak AR binding and weak 5α-reductase inhibition and its weak androgenic activity, since agonistic activity may develop more distinctly in rapidly growing tumors than in normal growing ventral prostates, and antagonistic activity may develop more effectively in ventral prostate having high 5α-reductase than in tumors having low 5α-reductase.

As a treatment for prostatic cancer, it is proposed that an antiandrogen such as flutamide which has no agonistic activity is desirable [5]. Although anthrasteroid **1**, which has modified androstane skeleton, acquires remarkable antagonistic activity, it still keeps weak agonistic activity. The modifications that make O–O distance shorter than those of the androgens might decrease the agonistic activity. Such modification studies aiming to design a pure antiandrogen are in progress in our laboratories.

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REFERENCES

1. Neumann F., Gräf K.-J., Hasan S. H., Schenck B. and H. Steinbeck.: Central actions of antiandrogens. In *Androgens and Antiandrogens* (Edited by L. Martini and M. Motta). Raven Press, New York (1977). p. 163.

2. Nakayama R., Hiraga K. and Miki T.; Mechanism of action of a steroidal anti-androgen TSAA-291, 16β-ethyl-17β-hydroxyestr-4-en-3-one. In *Hormone Antagonists* (Edited by M. K. Agarwal). de Gruyter, Berlin (1982) p. 269.
3. Suzuki T., Horibe I., Kato G., Uchida K. and Takeda K.: Modified steroids and their hormonal activities. *6th French–Japanese Symposium on Medicinal and Fine Chemistry*, Tours, France (1987) p. 86 (Abstr.).
4. Mineshita T. and Yamaguchi K.: An androgen-dependent tumor derived from a hormone-independent spontaneous tumor of a female mouse. *Steroids* **4** (1964) 815–830.
5. Luthy I. A., Begin D. J. and Labrie F.: Androgenic activity of synthetic progestins and spironolactone in androgen-sensitive mouse mammary carcinoma (Shionogi) cells in culture. *J. Steroid Biochem.* **31** (1988) 845–852.
6. Bruchovsky N.: The metabolism of testosterone and dihydrotestosterone in an androgen-dependent tumour. *Biochem. J.* **127** (1972) 561–575.
7. Yamaguchi K., Kasai H., Mineshita T., Kotoh K. and Matsumoto K.: 5α-Reduction and binding of testosterone in androgen-dependent and -independent mouse mammary tumors. *Endocrinology* **95** (1974) 1424–1430.
8. Shida K., Yamanaka H., Koya A., Wakabayashi K., Mori H., Shibata K. and Shimazawa E.: Action of a novel nonsteroidal antiandrogen, AA560. *Endocr. Jap.* **27** (1980) 69–76.
9. Mori H., Shibata T., Ohuchi R., Yamakoshi N., Kurata S., Shimazawa E., Meshitsuka, K. and Mitsueda, E.: Anilide derivatives. Japan Patent 128329 (1977) 299–334.
10. Kamata S., Haga N., Mitsugi T., Kondo E., Nagata W., Nakamura M., Miyata K., Odaguchi K., Shimizu T., Kawabata T., Suzuki T., Ishibashi M. and Yamada F.: Aldosterone antagonists. 1. Synthesis and biological activities of 11β,18-epoxypregnane derivatives. *J. Med. Chem.* **28** (1985) 428–433.
11. Munck A.: General aspects of steroid hormone-receptor interactions. In *Receptors and Mechanism of Action of Steroid Hormones Part I* (Edited by J. R. Pasqualini). Dekker, New York (1976) p. 1.
12. Noguchi S., Nishizawa Y., Nakamura N., Uchida N., Yamaguchi K., Sato B., Kitamura Y. and Matsumoto K.: Growth-stimulating effect of pharmacological doses of estrogen on androgen-dependent Shionogi Carcinoma 115 *in vivo* but not in cell culture. *Cancer Res.* **47** (1987) 263–268.
13. Nakamura N., Yamanishi H., Lu J., Uchida N., Nonomura N., Matsumoto K. and Sato B.: Growth-stimulatory effects of androgen, high concentration of glucocorticoid or fibroblast growth factor on a cloned cell line from Shionogi carcinoma 115 cells in a serum-free medium. *J. Steroid Biochem.* **33** (1989) 13–18.
14. Moore R. J. and Wilson J. D.: Reduced nicotinamide adenine dinucleotide phosphate: Δ^4-3-ketosteroid 5α-oxidoreductase (rat ventral prostate). *Meth. Enzym.* **36** (1975) 466–474.
15. Lineweaver H. and Burk D.: The determination of enzyme dissociation constants. *J. Am. Chem. Soc.* **56** (1934) 658–666.
16. Lowry O. H., Rosebrough N. J., Farr A. L. and Randall R. J.: Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193** (1951) 265–275.
17. SYBYL. Molecular Modeling Software, Version 5.3, Tripos Associates, St Louis, Mo.
18. Jensen E. V.: Intracellular localization of estrogen receptors: implications for interaction mechanism. *Lab. Invest.* **51** (1984) 487–488.
19. King R. J. B.: An overview of molecular aspects of steroid hormone action. In *Hormones and their Actions Part I* (Edited by B. A. Cooke, R. J. B. King and H. J. van der Molen). Elsevier/North-Holland, Amsterdam (1988) 29.
20. Parker M. G.: Gene regulation by steroid hormones. In *Hormones and their Actions Part I* (Edited by B. A. Cooke, R. J. B. King and H. J. van der Molen). Elsevier/North-Holland, Amsterdam (1988) p. 39.
21. Duax W. L., Griffin J. F., Weeks C. M. and Wawrzak Z.: The mechanism of action of steroid antagonists: insights from crystallographic studies. *J. Steroid Biochem.* **31** (1988) 481–492.
22. Hendry L. B.: Stereochemical complementarity of DNA and steroid agonists and antagonists. *J. Steroid Biochem.* **31** (1988) 493–523.
23. Kirchhoff J., Soffie, M. and Rousseau G. G.: Differences in the steroid-binding site specificities of rat prostate androgen receptor and epididymal androgen-binding protein ABP. *J. Steroid Biochem.* **10** (1979) 487–497.
24. Sudo K., Yoshida K. and Nakayama R.: Specific interaction of radioactive anti-androgen TSAA-291 with androgen receptor in rat prostates. *Acta Endocr. (Copenh.)* **100** (1982) 473–480.
25. Liao S., Liang T., Fang S., Castañeda E. and Shao T.-C.: Steroid structure and androgenic activity. *J. Biol. Chem.* **248** (1973) 6154–6162.
26. Shimazaki J., Ohki Y., Koya A. and Shida K.: Inhibition of nuclear testosterone 5α-reductase in rat ventral prostate by estrogens and anti-androgens. *Endocr. Jap.* **19** (1972) 585–588.
27. Roberts P. J., Pettersen R. C., Sheldrick G. M., Isaacs N. W. and Kennard O.: Crystal and molecular structure of 17β-hydroxyandrost-4-en-3-one (testosterone). *J. Chem. Soc. Perkin* **II** (1973) 1978–1984.
28. Courseille C., Precigoux G., Leroy F. and Busetta B.: 17β-Hydroxyandrostane-3-one, $C_{19}H_{30}O_2$. *Cryst. Struct. Commun.* **2** (1973) 441–446.