14α-Hydroxyandrost-4-ene-3,6,17-trione as a Mechanical-Based Irreversible Inhibitor of Estrogen Biosynthesis

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Various derivatives of androst-4-ene-3,17-dione derived from microbial transformation were evaluated as inhibitors of human placental aromatase. 14α-Hydroxyandrost-4-ene-3,6,17-trione was the most potent inhibitor showing a time-dependent, pseudo-first-order inactivation of aromatase in the presence of reduced nicotinamide adenine dinucleotide phosphate with apparent K_i of 1.3 μM and K_{inact} of 0.23 min^{-1}.

This compound also inhibited aromatase in rat ovary and suppressed serum estradiol levels in *in vivo* experiments.

Keywords aromatase; time-dependent-inhibition; 14α-hydroxyandrost-4-ene-3,6,17-trione; 6-keto steroid; androst-4-ene-3-one derivative; rat ovarian aromatase; serum estradiol suppression

Introduction

Various derivatives of androst-4-ene-3,17-dione (AD), the natural substrate for aromatase (estrogen synthetase) have been evaluated as inhibitors of estrogen biosynthesis.[1-3] Such compounds having aromatase inhibition activity are of potential clinical value for controlling estrogen mediated events, such as ovulation and the growth of estrogen-dependent tumors.[4] Some of the steroidal aromatase inhibitors were reported to show mechanical-based enzyme-activated irreversible inhibition.[5-7] These so-called "suicide" inhibitors might be very effective in clinical applications.

Studies on the structural features of AD derivatives as an inhibitor indicated that a hydroxy or keto substituent introduced into the AD molecule plays an important role in the inhibiting function.[1]

During our research program to develop a new steroidal inhibitor for aromatase, we found that 14α-hydroxyandrost-4-ene-3,6,17-trione (14OH-AT) produced by microbial transformation of AD is a very potent aromatase inhibitor *in vitro*.[8]

We report here the characterization of this compound as a mechanical-based aromatase inhibitor and an evaluation of its endocrine profile.

Experimental

Materials AD derivatives (14α-hydroxyandrost-4-ene-3,17-dione (**1**), 11α-hydroxyandrost-4-ene-3,17-dione (**2**), 7α,14α-dihydroxyandrost-4-ene-3,17-dione (**3**), 6β,14α-dihydroxyandrost-4-ene-3,17-dione (**4**) and 6β,11α-dihydroxyandrost-4-ene-3,17-dione (**5**)) were prepared according to the previous method using microbial transformation of AD.[8] Aminoglutethimide (AG) was obtained from Ciba-Geigy (Basel, Switzerland). Androst-4-ene-3,6,17-trione (AT) was synthesized by Jones oxidation of dehydroepiandrosterone.[9] [1β-^{3}H]AD (27.4 Ci/mmol) and [4-^{14}C]AD (52.0 mCi/mmol) were purchased from New England Nuclear (Boston, U.S.A.). Reduced nicotinamide adenine dinucleotide phosphate (NADPH) and other chemicals were obtained from Sigma Chemical Co. (St. Louis, U.S.A.). The E2 kit Daiichi was obtained from Daiichi Radio-isotope Laboratories.

Enzyme Preparation Human placental microsomal fraction was used as an aromatase enzyme.[10] The following preparation was carried out at 0—4 °C. Human placenta was obtained immediately upon delivery at term. Tissues were cut away from the fetal membrane and large blood vessels and washed with 1.1% KCl to remove excess blood. The pieces of washed tissue were homogenized in 67 mM potassium phosphate buffer, pH 7.5 with 5 mM dithiothreitol and 0.25 M sucrose in a mixer. The homogenate was centrifuged for 30 min at 10000 *g*. The pellet was discarded and the supernatant was centrifuged again at 104000 *g* for 60 min. The final pellet was resuspended in the buffer as in the above experiment without sucrose at a final concentration of 20 mg protein/ml and frozen in 0.5 ml aliquots at −80 °C until ready for use. Protein concentrations were determined by the method of Lowry *et al.*[11]

Aromatase Assay Aromatase activity was assayed using a tritiated water release assay originally developed by Thompson and Siiteri.[12] All incubations were carried out in 100 mM KCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 10 mM phosphate buffer pH 7.5, 0.5 mM NADPH, 2 μM of [1β-^{3}H]AD and 10 μl of placental microsomal fraction in 0.6 ml of the mixture. Incubations were performed at 37 °C for 30 min in air and terminated by addition of 5 ml of $CHCl_3$, followed by vortexing for 40 s. After centrifugation at 1470 *g* for 15 min, aliquots (0.1 ml) were removed from water phase and added to scintillation cocktails for determination of ^3H_2O production.[13]

Time-Dependent Inactivation Studies[5] Various concentrations of inhibitors were incubated with or without NADPH at 37 °C with the placental microsomes in the same incubation mixture of aromatase assay.

The incubations were terminated at a specified time by chilling in ice-water and 1 ml of charcoal solution (2.5% in the same buffer) was added. After centrifugation at 1400 *g* for 10 min, 0.5 ml of the supernatant was transferred into an aromatase assay tube, which was added with NADPH and [1β-^{3}H]AD at final concentrations of 0.5 mM and 2 μM, respectively.

Remaining activities were determined by ^3H_2O release as describedabove.

Animals Sprague-Dawley (SD) rats 50 to 55 d of age were used in this study. Animals were housed five per cage under a regimen of 12 h of light (lights on between 07:00 and 19:00) and 12 h of darkness in a temperature-controlled (23 ± 1 °C) environment. CRF-1 rat chow (Oriental Yeast Co. Japan) and water were given *ad libitum*.

Inhibition of Ovarian Estrogen Secretion[14] Female rats were given pregnant mares' serum gonadotropin (PMSG; 100 IU, s.c.) every other day for 12 d.[15] On the twelfth day of PMSG treatment, each rat was administered a test compound (50 mg/kg for s.c. or 100 mg/kg for *p.o.*) suspended in 0.3% hydroxy propyl cellulose, and others received vehicle only as control. After 3 h from s.c. or 6 h from *p.o.* administration, trunk blood was collected and the concentration of 17β-estradiol was then analyzed by radioimmunoassay using the E2 kit Daiichi. After collecting the blood, the ovaries were immediately removed and homogenized, and the aromatase activities were determined by measuring tritium released during incubation with [1β-^{3}H]AD as described above.

Bioassays[16] **1. Androgenic Bioassay** Male SD rats of 21 d of age were castrated. Beginning the next day, they were given 3, 10 or 30 mg of test sample suspended in 0.5 ml of sesame oil per rat with s.c. injection every morning for 6 d. On day 6, animals were weighed and killed by ether anesthesia. The seminal vesicles and prostates were removed, dissected free of other tissue and weighed; seminal vesicle and prostate weights were expressed as gram of organ per 100 g of body weight (%).

2. Estrogenic Bioassay Female SD rats 21 d of age were given 3, 10 or 30 mg of test sample with s.c. injection for 3 d as above. On day 4, animals were weighted and killed by ether anesthesia. The uteri were removed, dissected free of adhering non-uterine tissue and weighed; uterus weight was expressed as gram of uterus per 100 g of body weight (%).

Results and Discussion

Aromatase inhibitory activities of various steroids

TABLE I. Inhibition of Aromatase Activities by Various Androstenedione Derivatives

Inhibitors	Inhibition (%)	
	100 μM	10 μM
14α-Hydroxyandrost-4-ene-3,17-dione (**1**)	69.5	29.5
11α-Hydroxyandrost-4-ene-3,17-dione (**2**)	32.3	3.8
Androst-4-ene-3,11,17-trione (**6**)[a]	54.3	8.3
7α,14α-Dihydroxyandrost-4-ene-3,17-dione (**3**)	64.2	21.2
6β,14α-Dihydroxyandrost-4-ene-3,17-dione (**4**)	52.9	16.0
14α-Hydroxyandrost-4-ene-3,6,17-trione (**7**)[a]	95.3	78.5
6β,11α-Dihydroxyandrost-4-ene-3,17-dione (**5**)	4.1	1.4
Androst-4-ene-3,6,11,17-tetraone (**8**)[a]	52.7	6.1
Aminoglutethimide (AG)	50.0	5.1
Androst-4-ene-3,6,17-trione (AT)	96.3	88.4

$$\text{Inhibition (\%)} = \left[1 - \left(\frac{\text{aromatase activit with inhibitor}}{\text{aromatase activity without inhibitor}}\right)\right] \times 100 .$$

a) CrO_3 oxidation product of each of the above hydroxy compounds.

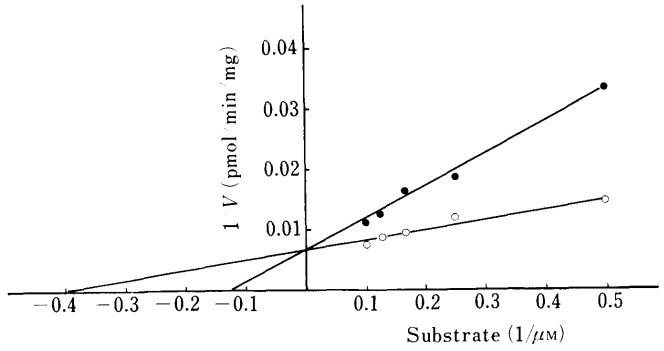

Fig. 1. Lineweaver–Burk Plot for 14OH-AT

14OH-AT 10 μM (●), no inhibitor (○).

obtained by microbial transformation of AD with *Acremonium strictum*[8] and their CrO_3 oxidation products are shown in Table I. AG and AT are also listed for comparison.

The 14α-hydroxy AD (**1**) showed relatively high potency, compared to 11α-hydroxy AD (**2**), on aromatase inhibition. An introduction of the second hydroxy group into the 7α- or 6β-position of compound **1** did not significantly change the inhibition activity. On the other hand, the 6β,11α-dihydroxy steroid was a very weak inhibitor.

These findings indicate that the introduction of a hydroxy moiety at 14α-position of AD has no significant effect on its binding property with the aromatase enzyme.

AD derivatives with newly introduced keto moiety(s) showed better inhibitory action than the corresponding hydroxy derivatives; this was particularly evident in a comparison of compounds **2**, **4** and **5** with their CrO_3 oxidation products (compounds **6**, **7** and **8**, respectively).

The most potent aromatae inhibitor among the test compounds evaluated in this study was 14OH-AT (**7**), and this was studied further to characterize the nature of its interaction with the aromatase enzyme.

Aromatization was measured at 10 μM of the inhibitor 14OH-AT in the presence of increasing concentrations of the AD substrate. The Lineweaver–Burk plot (Fig. 1) of the results yielded lines with a common intersection on the ordinate indicating its competitive type inhibition.

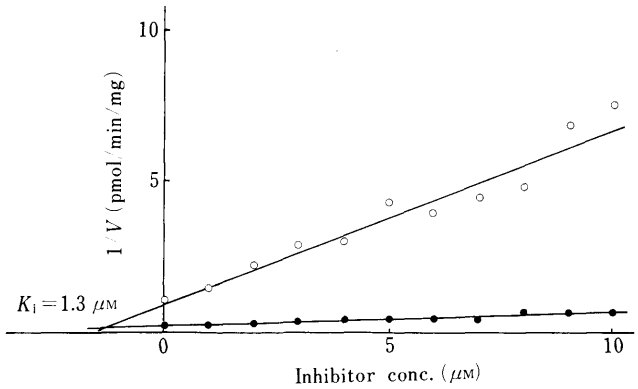

Fig. 2. Dixon Plot to Determine the Apparent Inhibition Constant K_i for 14OH-AT

The assay contained 0.1 μM (○) and 1 μM (●) of AD.

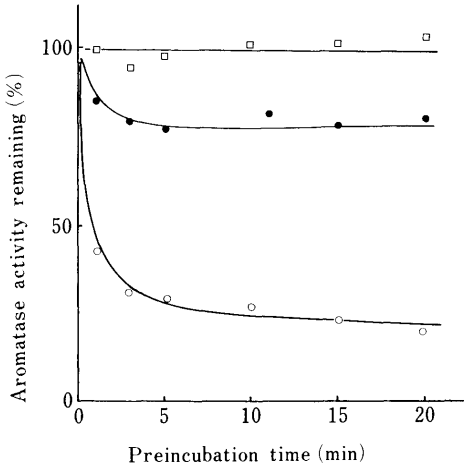

Fig. 3. Time-Dependent Loss of Aromatase Activity by 14OH-AT

14OH-AT with NADPH (○), 14OH-AT without NADPH (●), no inhibitor (□).

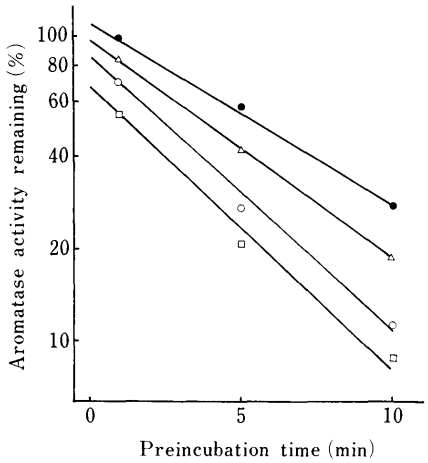

Fig. 4. Time-Dependent Inhibition in Aromatase Activity by 14OH-AT

14OH-AT concentrations: 75 nM (●), 150 nM (△), 300 nM (○), 750 nM (□).

Aromatization was also analyzed at several concentrations of the inhibitor in the presence of 0.1 and 1 μM of the substrates.

The Dixon plot of this experiment also showed 14OH-AT to be a competitive inhibitor with an apparent K_i of 1.3 μM (Fig. 2). This K_i value was almost the same as that of AT

TABLE II. Suppression of Ovarian Aromatase Activities and Estradiol Levels in Trunk Blood

	Control	AG	AT	14OH-AT
Aromatase activity[a]				
s.c. (50 mg/kg)	21.3±1.1	17.9±2.0	10.7±3.3[c]	11.8±2.3[c]
p.o. (100 mg/kg)	22.7±1.2	20.3±1.9	13.8±2.1[c]	12.7±1.1[c]
Estradiol level[b]				
s.c. (50 mg/kg)	0.572±0.079	0.250±0.071[d]	0.246±0.068[d]	0.262±0.049[d]
p.o. (100 mg/kg)	0.522±0.072	0.268±0.048[d]	0.258±0.064[d]	0.299±0.038[d]

Each value represents mean±S.D. ($n=10$). *a*) pmol/mg/min. *b*) ng/ml. *c*) $p<0.01$; *d*) $p<0.05$.

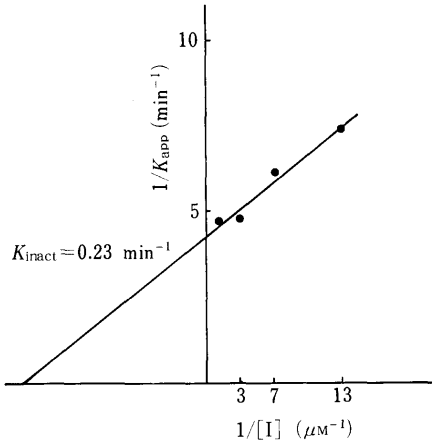

Fig. 5. Concentration-Dependent Inhibition in Aromatase Activity by 14OH-AT

($K_i=1.25\ \mu M$).[17] This indicates that a 14α-hydroxy moiety introduced into AT does not affect its inhibitory activity.

The next experiments were performed to examine whether or not 14OH-AT causes a time-dependent inactivation of aromatase, as reported for several steroidal inhibitors.[6,7,10] 14OH-AT was incubated with aromatase in the presence of NADPH in air and the remaining aromatase activity was measured.

Analysis of the data by the method of Kitz and Wilson[18] demonstrated that the increased inhibition observed with increased preincubation time followed the expected pseudo-first order kinetics (Figs. 3 and 4).

As shown in Fig. 3, 14OH-AT did not cause any significant inactivation in the absence of NADPH, while the remaining activity was decreased by about 70% in the presence of the cofactor. Thus, it is concluded that the time-dependent inactivation observed in this study is principally a mechanical-based irreversible inhibition rather than an affinity-labeling inhibition which does not required NADPH as a cofactor. This type of irreversible inhibition is referred to as "suicide" inhibition. With increasing concentrations of inhibitor, increasing K_{app} values were obtained for 14OH-AT.

A double reciprocal plot of K_{app} *vs.* inhibitor concentration yielded K_{inact} value of 0.23 min^{-1} as an inactivation constant (Fig. 5). This K_{inact} value is very near that of AT (0.24 min^{-1}),[10] indicating that an additional 14α-hydroxy moiety in the AD molecule does not change its mechanism of enzyme inhibition.

Thus, the aromatase inhibition mechanism caused by this 14OH-AT was assumed to have the same characteristics as AT.

TABLE III. Androgenic Bioassay of 14OH-AT

Compound	mg/rat	Seminal weight (%)[a]	Prostate weight (%)[a]
Control		0.0179±0.0032	0.0117±0.0030
TP			
	0.01	0.0465±0.0095[b]	0.0669±0.0063[c]
	0.1	0.1572±0.0434[c]	0.1293±0.0241[c]
	1.0	0.2366±0.0297[c]	0.1559±0.0185[c]
AD			
	0.1	0.0392±0.0041	0.1054±0.0141[c]
	1.0	0.1077±0.0266[c]	0.1465±0.0249[c]
	10.0	0.2465±0.0283[c]	0.1654±0.0175[c]
AT			
	3.0	0.0371±0.0051	0.0915±0.0139[c]
	10.0	0.0875±0.0176[c]	0.1055±0.0071[c]
	30.0	0.1745±0.0158[c]	0.1268±0.0163[c]
14OH-AT			
	3.0	0.0232±0.0028	0.0145±0.0035
	10.0	0.0107±0.0027	0.0146±0.0044
	30.0	0.0151±0.0024	0.0143±0.0060

Each value represents mean±S.D. ($n=6$). TP: testosterone propionate. *a*) Gram of organ per 100 g of body weight. *b*) $p<0.05$; *c*) $p<0.01$.

TABLE IV. Estrogenic Bioassay of 14OH-AT

Compound	mg/rat	Uterine weight (%)[a]
Control		0.0647±0.0074
Estradiol		
	0.01	0.0843±0.0060[b]
	0.1	0.1471±0.0139[b]
	1.0	0.2064±0.0080[b]
14OH-AT		
	3.0	0.0671±0.0089
	10.0	0.0646±0.0147
	30.0	0.0607±0.0072

Each value represents mean±S.D. ($n=6$). *a*) Gram of uterus per 100 g of body weight. *b*) $p<0.01$.

The above results prompted us to evaluate a number of *in vivo* experiments with 14OH-AT in order to determine its endocrine profile and pharmacological potency. According to preliminary results, the maximum aromatase inhibition was observed at 3 and 6 h after s.c. and *p.o.* administration of 14OH-AT (data not shown).

Table II shows the results of suppression of ovarian aromatase activity and serum estradiol levels caused by single administration *via* s.c. (50 mg/kg) or *p.o.* (100 mg/kg) of 14OH-AT in female rats, and the results with AG and AT are also listed for comparison. The estradiol levels in the serum of trunk blood were equally suppressed by 14OH-AT, AT and AG in both s.c. and *p.o.* administrations, whereas the aromatase activities in the ovaries were blocked

by 14OH-AT and AT to a greater extent than AG both in s.c. and *p.o.* administrations (Table II).

From the above results, the *in vivo* biotransformation and bioavailability of 14OH-AT might be the same as those of AT.

When the substrate analogue is used as an enzyme inhibitor for pharmaceutical purposes, there is a possibility that it also possesses physiological functions of the parent substrate. AD acts as androgen *in vivo*, although its activity is almost ten times lower than that of testosterone. Table III shows the androgenic activity of 14OH-AT in male rats, and those of testosterone propionate, a standard androgen, AT and AD are also listed for comparison. The estrogenic activity of 14OH-AT was also examined in female rats (Table IV). 14OH-AT has neither androgenic nor estrogenic activity in the present bioassay systems using rats of either sex. However, it should be noted that AT showed androgenic activity on both seminal vesicle and prostate weights almost ten times lower than that of AD. This indicates that a 14α-hydroxy moiety of 14OH-AT plays an important role in the almost complete disappearance of the androgenic property.

The bioavailability of 14OH-AT is suggested to be the same as AT, so its affinity to the androgenic receptor is supposed to be much weaker than AT.

Thus, 14OH-AT is a potent aromatase inhibitor both *in vitro* and *in vivo* and has almost no androgenic activity, encouraging further development of this compound as a potential medicinal agent for the treatment of estrogen-dependent disease states such as breast and endometrial cancers.

Further studies are in progress to establish its anti-cancer profiles and to define its pharmacological potential.

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