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INHIBITORY EFFECT OF A NEW ANDROSTENEDIONE DERIVATIVE, 14α-HYDROXY-4-ANDROSTENE-3,6,17-TRIONE (14α-OHAT) ON AROMATASE ACTIVITY OF HUMAN UTERINE TUMORS*

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Summary—The development of human uterine estrogen-dependent tumors is considered to be closely related to estrogen biosynthesis. This study examined whether or not 14α-hydroxy-4-androstene-3,6,17-trione (14α-OHAT), a new 4-androstene-3, 17-dione derivative synthesized microbiologically, inhibits estrogen biosynthetase (aromatase) activities of human uterine tumors (i.e. uterine endometrial cancer, uterine leiomyoma and uterine adenomyosis tissues).

14α-OHAT inhibited aromatase activity in all uterine tumors, dose-dependently (0.1–10 μM).

Moreover, 14α-OHAT did not show the binding affinity to rabbit uterine cytosol-sex steroids, and it was not converted to estrogen in human placental preparations.

Thus, 14α-OHAT, an aromatase inhibitor, may be useful clinically as an endocrine chemotherapy for peri- or post-menopausal women with uterine estrogen-dependent tumors.

INTRODUCTION

Various C19-steroidal derivatives, as irreversible inhibitors for estrogen biosynthetase (aromatase) which convert androstenedione (or testosterone) to estrogen with 3 molecules of NADPH and 3 molecules of oxygen [1, 2], have been designed and synthesized. These steroidal substrates are well known to effectively inhibit aromatase activity in human placental preparations. Some of them are expected to be useful in endocrine-chemotherapy for advanced malignant estrogen-dependent tumors (i.e. breast cancer) [3, 4]; because, in women with breast cancer or uterine endometrial cancer, the tumor itself has aromatase activity and thus may be an additional source of estrogen for the developing tumor. Also, as aromatase inhibitors lower circulating estrogen levels, they might be used in other hormonal diseases resulting from hyperestrogenism.

Recently, Yoshihama *et al.*[5] reported that 14α-hydroxy-4-androstene-3,6,17-trione (14α-OHAT), a new derivative transformed microbiologically from 4-androstene-3,17-dione (AD), demonstrates an irreversible inhibitory effect for aromatase in human placental microsomes (Fig. 1). In order to further this study, we tried to demonstrate whether or not 14α-OHAT binds with sex steroid receptors, whether or not 14α-OHAT is aromatized to estrogen in human placental preparations, and whether or not 14α-OHAT effectively suppresses aromatase activity of human uterine tumor tissues.

MATERIALS AND METHODS

Steroids

[6,7-^{3}H]estradiol-17β ([^{3}H]E_2, SA47.9 Ci/mmol), 17α,21-dimethyl-19nor-pregna-4,9-diene-3,20-dione [17α-methyl-^{3}H] ([^{3}H]promegestone, SA 86 Ci/mmol), [1,2-^{3}H]17β-hydroxy-5α-androstan-3-one ([^{3}H]DHT, SA 40 Ci/mmol), 17β-hydroxy-[17α-methyl-^{3}H]estra-4,9,11-triene-3-one ([^{3}H]methyltrienolone, SA 87 Ci/mmol) and [1β-^{3}H]androstenedione ([1β-^{3}H]AD, SA 27.8 Ci/mmol) were purchased from NEN Research Products (Boston, Mass). Non-labeled steroids were purchased from Sigma Chemical Company (St Louis, Mo.).

The possibility of 14α-OHAT aromatization in human placental preparation

Placental tissue was obtained after normal vaginal delivery at term and blood was removed with saline. The placental tissue was minced, homogenized in ice-cold 1/15 M-bisodium phosphate buffer (pH 7.4) with a Polytron homogenizer, and centrifuged at 800 *g* for 10 min. The supernatant fraction was centrifuged at 9500 *g* for 30 min. The supernatant

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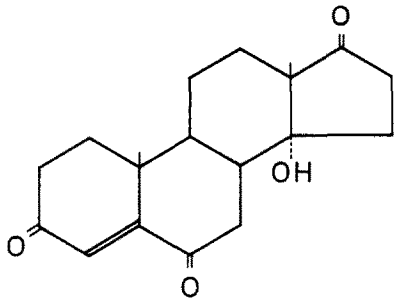

Fig. 1. The chemical structure of 14α-hydroxy-4-androstene-3,6,17-trione (14α-OHAT).

obtained was further centrifuged at 105,000 *g* for 60 min. The pellets were used as microsomal fraction in this experiment. 14α-OHAT (1 mg in 0.2 ml of propylene glycol) was added to the microsomal fraction (100 mg protein in 10 ml of 1/15 M-phosphate buffer) with 10 mg of NADPH (Sigma Chemical Co.).

Two samples of the mixture were incubated at 37°C in air for 20 and 120 min, respectively. The reaction was stopped with 3 volumes of ethyl acetate. Steroids were extracted 3 times with ethyl acetate. The ethyl acetate was evaporated and the extract was resuspended in 1 ml of methanol. The sample obtained was chromatographed on a Bio-Rad AG1-X2 (Bio-Rad Labs, Richmond, Calif.) to obtain the phenolic steroid fraction [6]. The phenolic steroid fraction was submitted to high pressure liquid chromatography (Spectraphysics, San Jose, Calif.) HPLC conditions: detected u.v. 230 nm, solvent; acetonitrile: water = 1:2, flow rate 1 ml/min, column ODS (Senshue pak, Tokyo) 4.6 × 150 mm.

The binding affinity of 14-OHAT to uterine cytosol-^{3}H-labeled steroid hormone binding

The uterine cytosol fraction used was prepared from immature female rabbits (approx. 800 g body wt). The rabbits were administered E_2 (intramuscular 50 μg/day × 7 days) and 2 days after final administration they were killed. The rabbit uterine tissue obtained was minced and homogenized with 5 volumes/weight TEG buffer [10 mM Tris, 1.5 mM ethylenediaminetetra-acetic acid (EDTA) and 10% glycerol, pH 7.4]. The homogenate was centrifuged at 800 *g* for 10 min and then the supernatant obtained was further centrifuged in nitrocellulose tubes at 150,000 *g* for 1 h to yield the supernatant fluid (a cytosol fraction). The cytosol fraction was exposed in the same volume of 0.1% dextran-coated charcoal (DCC; 0.001% dextran and 0.1% charcoal) solution suspended in TEG buffer at 4°C for 1 h.

5 nM [^{3}H]E_2, 5 nM [^{3}H]promegestone or 5 nM [^{3}H]methyltrienolone, with increasing concentrations of 14α-OHAT or each related unlabeled steroid (from zero to 10^3-fold excess) with 0.2 ml of cytosol fraction was incubated at 20°C for 2 h in triplicate. Unbound hormones were removed by 0.25% DCC (incubation time; 30 min at 4°C). The radioactivity in bound form was determined in aqueous counting scintillant II (Amersham, Arlington Heights, Ill.) with a Packard Tri-Carb No. 460 automatic scintillation system (Packard Instrument Company, Downers Grove, Ill.).

Inhibitory effect of 14α-OHAT on aromatase activity of human uterine tumors

Uterine endometrial cancer tissues were obtained from a 64-yr-old patient (height 152 cm, weight 50 kg) with endometrial cancer (G_2 type) and a 52-yr-old patient (height 156 cm, weight 48 kg) with endometrial cancer (G_1 type) who had undergone a radical hysterectomy. Uterine leiomyoma tissues were obtained from a 46-yr-old patient (height 156 cm, weight 58 kg) and a 38-yr-old patient (height 159 cm, weight 68 kg). Uterine adenomyosis tissues were from a 42-yr-old patient (height 162 cm, weight 55 kg) and a 40-yr-old patient (height 158 cm, weight 51 kg) after hysterectomy. The breast cancer tissue was obtained after surgical treatment of a 38-yr-old patient (height 159 cm, weight 68 kg) with breast cancer (papillotubular adenocarcinoma). All tissues used for this experiment were diagnosed pathologically before tissue incubation. The tissue (approx. 1 g wet wt) was minced and homogenized in 5 ml of ice-cold 1/15 M-phosphate buffer solution (pH 7.4) with a Polytron homogenizer. The homogenate was centrifuged at 800 *g* for 10 min and the supernatant fraction (crude microsomes) was used for this experiment. Various concentrations (0, 0.1, 1.0 and 10 μM) of 14α-OHAT were added to 0.5 ml of the crude microsomal fraction. Each mixture was incubated with [1β-^{3}H]AD (100 pmol) and NADPH (0.5 mg) at 37°C for 2 h in air. The incubation for each tissue sample was carried out in duplicate. The enzyme reaction was stopped by the addition of 10% trichloro-acetic acid (0.4 ml) and the mixture was extracted with chloroform (3 ml). The residual aqueous phase after extraction was subjected to Amberlite XAD-II resin-charcoal column chromatography. The amount of ^3H_2O released during incubation was regarded as aromatase activity in the uterine tissue preparation. Also, aromatase activity in the tissue sample was calculated by subtracting the levels of radioactivity determined in the control samples (no tissue blank sample). Protein in the tissue samples was measured by employing a Bio-Rad Protein Assay Kit (Bio-Rad Lab, Richmond, Calif.).

RESULTS

No amount of estrogen was detected (detection limit; under 100 ng) by HPLC in either the 20 min- or 120 min-incubation experiments with 14α-OHAT and human placental microsomes. Aromatase activity was 28 pmol/min/mg protein in the placental microsomes used. In our method used for this experiment, it was decided that the degree of aromatization

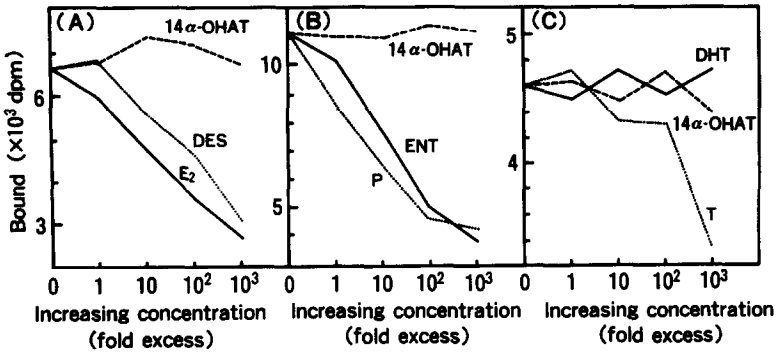

Fig. 2. Effect of increasing concentration of 14α-OHAT or related unlabeled steroids on [^{3}H]estradiol (A), [^{3}H]promegestone (B) and [^{3}H]methyltrienolone (C) protein binding in immature rabbit uterine cytosol. Each value represents a mean of triplicate determinations. 14α-OHAT: 14α-hydroxy-4-androstene-3,6,17-trione; DES: diethylstilbestrol; E_2: estradiol-17β; ENT: 17α-ethyl-19-nortestosterone; P: progesterone; DHT: dihydrotestosterone; T: testosterone.

for 14α-OHAT was under 0.1% and thus it was not transformed to an estrogen (i.e. estrone, estradiol-17β or 14α-hydroxyestrone) in human placental microsomes.

The binding affinity of 14α-OHAT to sex steroid hormone receptors was examined by using rabbit uterine cytosol. Rabbit uterine cytosol [^{3}H]E_2 binding was inhibited by an excess of unlabeled E_2 or diethylstilbestrol (DES). However, [^{3}H]E_2 binding was not inhibited by a 10^3-fold excess of 14α-OHAT. On the other hand, though uterine cytosol [^{3}H]promegestone binding was significantly inhibited by a 10^2-fold excess of progesterone or 17α-ethynyl-19-nortestosterone (ENT), its binding activity was not inhibited by a 10^3-fold excess of 14α-OHAT.

In immature rabbit uterus, the content of cytosol [^{3}H]methyltrienolone binding protein was speculated to be rather small, but cytosol [^{3}H]methyltrienolone binding was not inhibited at all by adding a 10^3-fold excess of 14α-OHAT (Fig. 2).

These results suggest that 14α-OHAT does not bind to cytosolic steroid hormone receptors [estrogen receptor (ER), progestogen receptor (PR) and androgen receptor (AR)] in rabbit uterus.

Aromatase activity of uterine endometrial cancer tissues from a 64-yr-old patient and a 52-yr-old patient was 0.99 and 1.69 pmol/h/100 mg protein, respectively. Aromatase activity of uterine leiomyoma tissue from a 46-yr-old patient and a 38-yr-old patient was 0.73 pmol/h/100 mg protein and 0.39 pmol/h/100 mg protein, respectively. Aromatase activity of uterine adenomyosis tissue was 0.28 pmol/h/100 mg protein in a 42-yr-old patient and 0.16 pmol/h/100 mg protein in a 40-yr-old patient, respectively. Aromatase activity was determined under 0.01 pmol/h/100 mg protein in the control samples (a heat inactivated leiomyoma tissue and a leiomyoma tissue without NADPH). We have confirmed the presence of aromatase activity in human uterine leiomyoma [8], adenomyosis [9] and endometrial cancer tissues [10] with the double isotope technique using [6,7-^{3}H]AD. In samples of uterine leiomyoma, the results from the radiometric assay using [1β-^{3}H]AD were at almost the same level [7] as co-crystallization data from the double-isotope technique using [6,7-^{3}H]AD. We have therefore concluded that the radiometric assay is valid for the quantitative inhibition assay of uterine tumor tissues. The aromatase activity in human uterine tumor tissues was decreased dose-dependently with 14α-OHAT (Fig. 3). One micromole, especially, of 14α-OHAT suppressed aromatase activity remarkably. However, a significant difference in aromatase inhibition by 14α-OHAT among uterine endometrial cancer, uterine leiomyoma and uterine adenomyosis tissues was not observed in this experiment. On the other hand, aromatase activity in breast cancer tissue was 1.97 pmol/h/100 mg protein. The degree of aromatase inhibition in breast cancer tissue was not much greater than those of uterine tumors with concentrations of 14α-OHAT over 1.0 μM.

Thus these results suggest that 14α-OHAT significantly suppresses aromatase activity of tumors without stimulating estrogen action in patients with uterine estrogen-dependent tumors.

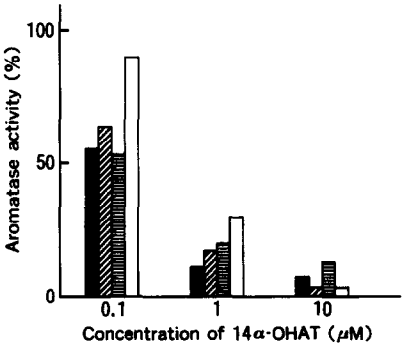

Fig. 3. Concentration-dependent aromatase inhibition of 14α-OHAT in various human uterine tissue preparations. Each bar represents a mean percent value for aromatase activity in the tissue sample in the presence of 14α-OHAT. Uterine leiomyoma tissue ■, $n = 2$; uterine adenomyosis tissue ▨, $n = 2$; uterine endometrial cancer tissue ▤, $n = 2$; breast cancer tissue □, $n = 1$.

DISCUSSION

When an irreversible substrate for aromatase reaches the target tissue, it binds to active sites of the aromatase protein and irreversibly blocks the action of aromatase. In recent years, employing such theory many synthetic steroids resembling endognenous AD have been developed towards application in clinical trials for women with breast cancer [3, 4].

Uterine endometrial cancer, uterine leiomyoma and uterine adonomyosis are estrogen-dependent tumors, and rarely complicate with breast cancer. Especially, it is said that in Japan the appearance of uterine endometrial cancer is gradually increasing, as is breast cancer. These uterine tumors possess high aromatase activities compared to normal tissues [7–11].

Tseng *et al.*[11] and Yamaki *et al.*[10] have reported that AD aromatization ability in uterine endometrial cancer tissues is significantly higher compared to normal endometrium of the uterus. However, no differences between patients with or without endometrial cancer have been found for levels of total E_2, sex hormone binding globulin (SHBG), non-SHBG-bound E_2, or absolute free E_2 in blood [12, 13]. On the other hand, Calanog *et al.*[14] suggested that when a priming dose of 15 μCi of [7-^{3}H]AD was given to postmenopausal patients with or without endometrial cancer, the plasma concentration of AD and the instantaneous conversion of AD to E_1 was increased in patients with cancer (mean $\pm$ SE, 26.6 ± 1.9 pg/ml; $n = 14$) as compared to postmenopausal control subjects (mean $\pm$ SE, 10.3 ± 0.31 pg/ml; $n = 5$). Moreover, they reported that the percentage of estrone derived from AD was elevated in the cancer groups. Uterine leiomyoma is a benign estrogen-dependent tumor and its tumor volume is reduced with a decrease in circulating estrogen levels. We previously reported that human uterine leiomyoma tissues have a greater ability to aromatize AD to E_1 and E_2 than normal myometrial tissues [8], and the aromatase activity of uterine leiomyoma was significantly suppressed by aminoglutethimide, which is well known as an aromatase inhibitor [15]. Especially, aromatase activity tends to be higher in the order of: center, middle and outside in myoma nodes [8]. Recently, our group [9] reported that aromatase activity in uterine adenomyosis tissue was also suppressed by adding danazol, and that the danazol-effect on tumor reduction may be due to its aromatase-inhibition. Thus, we have speculated that the growth of uterine endometrial cancer, leiomyoma and adenomyosis is closely related to aromatase activity in these tumors, and that the tumor growth may be suppressed by the administration of aromatase inhibitors.

14α-OHAT, a new AD derivative, is a synthetic steroid transformed from AD by a microorganism, Acremonium strictum NN106, and 14α-OHAT irreversibly suppressed aromatase activity in human placental microsomes [5]. Also, the degree of inhibitory placental aromatase activity by 14α-OHAT was much greater than aminoglutethimide. Our results obtained in this study indicate that 14α-OHAT, moreover, significantly suppresses the aromatase activity of uterine estrogen-dependent tumor without binding to ER, other than in case of breast cancer. However, in this study, we could not clarify whether or not 14α-OHAT is an irreversible inhibitor for aromatase in uterine tumor preparations, because the uterine tissue obtained was so small and its aromatase was too low to analyze the mechanism of inhibition.

Therefore, 14α-OHAT may be effective when clinically applied against uterine and breast estrogen-dependent tumors in peri- or post-menopausal women, by inhibiting the aromatase activity and by reducing tumor growth.

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