7 α -SUBSTITUTED ANDROSTENEDIONES AS EFFECTIVE *IN VITRO* AND *IN VIVO* INHIBITORS OF AROMATASE

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ABSTRACT

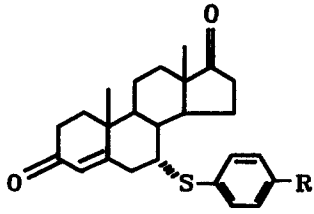
Research efforts over the past several years have focused on the synthesis of competitive and irreversible aromatase inhibitors and examination of these inhibitors in microsomal preparations, in cell culture, and in vivo. Several 7α-substituted androstenediones have demonstrated high affinity for placental aromatase, with apparent K_i's ranging from 1 to 30 nM. Inactivation of aromatase occurred following incubation with alkylating and enzyme-activated irreversible inhibitors. 7α-(4'-Amino)phenylthio-4-androstene-3,17-dione (7α-APTA) exhibits potent inhibitory activity of aromatase in the MCF-7 human mammary carcinoma cell line with an ED_{50} of approximately 25 nM. The inhibitor did not bind to the estrogen receptor of the cells in vitro nor induce levels of progesterone receptors in intact cells. In vivo studies of 7α-APTA in the DMBA-induced rat mammary carcinoma model resulted in 80% of the tumors responding completely or partially at doses of 25 and 50 mg/kg body wt/day. Thus, these 7α-substituted steroidal aromatase inhibitors are effective medicinal agents and may be useful for the treatment of estrogen-dependent breast cancer.

INTRODUCTION

Aromatase is the cytochrome P-450 enzyme complex responsible for estrogen biosynthesis in vivo. Inhibitors of this enzyme complex may be useful in controlling reproductive processes and in treating estrogen-dependent disease states such as breast and endometrial cancer, since estrogen production would be suppressed by these agents in all tissues including peripheral sites. The therapeutic efficacies of aromatase inhibitors such as 4-hydroxyandrostenedione and aminoglutethimide are being investigated and these agents have been shown to cause regression of hormone-dependent breast tumors in both rats (1-3) and humans (4,5).

Substitution at the 7α-position of androstenedione results in inhibitors of enhanced affinity for aromatase. 7α-(4'-Amino)phenylthio-4-androstene-3,17-dione, 7α-APTA (1), is among the most potent competitive inhibitors produced (6-10). Several irreversible and photoaffinity analogs of 7α-APTA (2-7) have also been prepared and exhibit good inhibition of microsomal aromatase.

Table 1. 7α-Substituted Aromatase Inhibitors



cmpd	name	R	K_i (nM)	K_{inact} (μM)
1	7-APTA	$-NH_2$	18	–
2	7-IPTA	$-I$	12	–
3	7-MeOPTA	$-OCH_3$	31	–
4	dimesylate	$-N(CH_2CH_2OSO_3CH_3)_2$	–	1.05
5	dichloro	$-N(CH_2CH_2Cl)_2$	–	9.77
6	bromoacetamide	$-NHCOCH_2Br$	–	32.90
7	azide	$-N_3$	1	0.88

Further development of this class of aromatase inhibitors is focusing on (a) the design and synthesis of new, more specific agents (11) and (b) further pharmacological evaluation of potent analogs in intact cell systems and in vivo animals containing estrogen-dependent tumors.

MATERIALS AND METHODS

Commercially available steroids were obtained from Steraloids (Wilton, NH). 7α-Substituted steroids were prepared as previously described (6-11). All chemicals were purchased from Aldrich Chemical Co. (Milwaukee, WI), radiolabeled steroids were purchased from New England Nuclear (Boston, MA), and biochemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

MCF-7 cells were obtained from the Ohio State University Cell Culture Service. A modified Eagle's minimum essential medium (MEM) supplemented with essential amino acids (1.5x), vitamins (1.5x), non-essential amino acids (2x), and L-glutamine (1x) was obtained in powdered form from GIBCO (Long Island, NY). The sterilized liquid media was prepared in the Ohio State University Comprehensive Cancer Center by dissolving the powder in water containing sodium chloride (0.487 g/L), pyruvic acid (0.11 g/L), sodium bicarbonate (1.5 g/L) and phenol red (0.01%), and the pH adjusted to 6.8. Fetal calf serum was obtained from KC Biological (Lenexa, KS). Steroids were removed from the fetal calf serum by two treatments with dextran-coated charcoal (12).

Female Sprague-Dawley rats (50-60 days old) were purchased from Harlan Industries, Inc. (Cumberland, IN). Animals were housed in metal cages containing ground corn cob (Anderson's, Maumee, OH), provided Purina Lab Chow and water ad libitum, and maintained in an AAALAC-accredited animal facility with a 12-h alternating light/dark cycle.

Biochemical Methods

Inhibitors were examined for aromatase inhibitory activity by monitoring the release of ^3H_2O from [1-^{3}H]androstenedione in placental microsomes under initial velocity conditions (8,11). Aromatase inactivation studies (11) were performed by incubating various concentrations of inhibitor (20-100 nM) with placental microsomes (2-3 mg protein), propylene glycol (100 µL) and NADPH (0.1 mM) in 0.1 M sodium phosphate buffer, pH 7.0, at 37°C. Aliquots (1.5 mL) were removed at various time periods (0-10 min) and immediately diluted 1:10 with cold buffer. The remaining enzyme activity was assayed by addition of the microsomal suspension to a solution of [1-^{3}H]androstenedione (0.5 µM, 200,000 dpm), propylene glycol (100 µL), NADP (1.8 mM), glucose-6-phosphate (2.85 mM), and glucose-6-phosphate dehydrogenase (4 units) in 0.1 M sodium phosphate buffer to a final volume of 3.6 mL and incubated at 37°C for 30 min. The assay was stopped by addition of 5 mL $CHCl_3$, vortexed for 20 sec, and centrifuged at 1250 xg for 5 min. Aliquots counted for ^{3}H dpm by LSC. Controls were run simultaneously with no inhibitor present. Inactivation studies in the absence of NADPH were performed in the same manner where NADPH is omitted in the initial incubation. Protection studies were carried out analogous to the inactivation studies with unlabeled 4-androstene-3,17-dione (0.3-0.5 µM) included with inhibitor in the initial incubation. Inactivation studies in the presence of nucleophilic trapping agents were carried out as above with cysteine or β-mercaptoethanol (0.5 mM) included with the inhibitor in the initial incubation.

Cell Culture Studies

MCF-7 mammary cells were grown in 150 cm^2 plastic flasks at 37°C in a modified Eagle's MEM culture medium (20 mL) containing 10% fetal calf serum. The aromatase inhibitors were added to cultures that were 90% confluent (approximately 1 x 10^7 cells) at concentrations of 10 pM to 1 μM in 10 μL ethanol. For all cell culture studies, experiments were carried out using triplicate flasks. Aromatase activity was determined using two methods: **(A)** [1,2,6,7-^{3}H]Testosterone (30 nM, 10 μCi) and unlabeled estradiol (10 nM, estrogen trap) were dissolved in 10 μL 95% ethanol and added to the cultures at the same time as the inhibitor. Control cultures did not receive inhibitor and blank samples contained [^{3}H]testosterone and unlabeled estradiol in medium only (no cells). At 6 h, the flasks were removed from the incubator, an aliquot of [4-^{14}C]estradiol (15,000 dpm) was added to the medium as an internal standard, cold 30% TCA (10 mL) was added to the medium to precipitate proteins, and the medium were extracted with ethyl acetate. The ethyl acetate was evaporated under nitrogen and the residue dissolved in 70% acetonitrile in water (1.0 mL). The isolation of [^{3}H]estradiol and [^{14}C]estradiol from each sample was accomplished by reverse-phase HPLC with a mobile phase of 40% acetonitrile in water. Fractions were collected and counted by LSC. The amount of [^{3}H]estradiol and [^{14}C]estradiol isolated was determined by liquid scintillation counting and the total estradiol produced in the cultures calculated. The percent inhibition was determined by dividing the total amount of estradiol formed in the particular inhibitor sample by the amount of estradiol formed in the uninhibited (control) samples. **(B)** [1-^{3}H]Androstenedione (30 nM, 2 μCi) and unlabeled estradiol (10 nM) were dissolved in 10 μL 95% ethanol and added to the cultures at the same time as the inhibitor. At 6 h, the flasks were removed and extracted three times with ethyl acetate. ^3H_2O in the aqueous medium layer was determined by LSC.

Tumored Animal Studies

Female rats (Sprague-Dawley, 50 days old) were gavaged with 20 mg of DMBA in 2 mL of sesame oil per rat (13). Rats were selected for the study when at least one tumor had a diameter of 2 cm, which was about 4 months after the administration of DMBA. The tumor volume is calculated using the equation $v = (4/3)\pi r_1^2 r_2$, where r_1 is the minor radius and r_2 is the major radius. The rats were divided into groups consisting of 7 animals with approximately the same number of tumors per rat and the same tumor volume per rat. 7α-APTA was examined at a dose of 25 and 50 mg/kg body wt/day. The compound was suspended in sesame oil (0.5 mL/injection) and each rat was injected subcutaneously daily. Rats in the control group received only sesame oil (0.5 mL daily). To determine the effects of co-treatment with estradiol, 7α-APTA (50 mg/kg) was dissolved in sesame oil (0.5 mL/injection) and each rat was injected subcutaneously daily for 3 weeks. Beginning at week 4, 7α-APTA (50 mg/kg) and estradiol (0.3 μg/kg) were dissolved in sesame oil (0.5 mL/injection) and each rat was injected subcutaneously daily for three more weeks. The rats were weighed twice a week and the number and volume of the tumors present determined over a 6-week period.

RESULTS

Enzyme-Activated Irreversible Inhibitor

Mechanism-based inhibitors of enzymes are considered to be effective agents in drug therapy (14). This type of inhibition involves enzymatic conversion of an inhibitor to a chemically-reactive species capable of irreversibly binding with the enzyme (15,16). These inhibitors offer advantages over competitive and affinity labeling inhibitors due to their high specificity and the irreversible nature of the inhibition produced. Mechanism-based (also referred to as enzyme-activated) inhibitors have been designed for aromatase (17-21), and several competitive inhibitors such as 4-hydroxyandrostenedione and 1,4,6-androstatriene-3,17-dione (ATD) were recently shown to produce inactivation of aromatase by an enzyme-catalyzed process (22-24).

Substitution of androstenedione at C-7α results in inhibitors of enhanced affinity for aromatase (6-10). The introduction of both a C_1-C_2 double bond and 7α-substitution on androstenedione would result in the compound 7α-(4'-amino)phenylthio-1,4-androstadiene-3,17-dione (7α-APTADD) and may provide a potent enzyme-activated inhibitor. This desired inhibitor was synthesized via a conjugate addition of p-aminothiophenol to ATD (Scheme I). Addition products at both C_7 (compound 9) and C_1 (compound 10) were formed in a ratio of 1:2.7 respectively (11).

Inhibition studies were conducted on 7α-APTADD under initial velocity conditions in order to determine its affinity for aromatase. 7α-APTADD 9 proved to be a potent inhibitor of aromatase with an apparent K_i of 9.9 ± 1.0 nM. In inactivation studies, 7α-APTADD 9

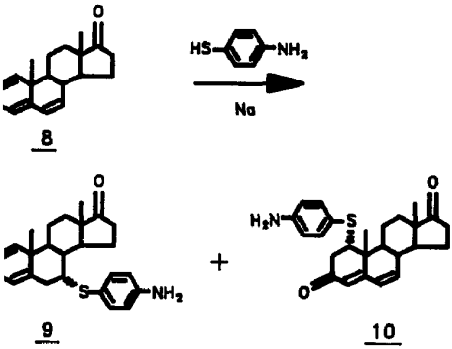
produced a rapid time-dependent, first-order inactivation of aromatase in the presence of NADPH (Fig. 1), while no inactivation of aromatase activity was observed in the absence of NADPH (Fig. 2). These results suggest that enzyme catalysis is necessary for the inactivation produced by 7α-APTADD. Varying concentrations of the substrate, androstenedione, in the incubation mixture containing enzyme, inhibitor (50 nM), and NADPH decreased inactivation by 7α-APTADD (Fig. 3). On the other hand, the addition of cysteine (0.5 mM) to the incubation mixture of enzyme, inhibitor (50 nM), and NADPH failed to protect aromatase from inactivation (Fig. 3), suggesting that the reactive inhibitor was not diffusing out of the active site.

Kinetic analysis of this inactivation process (11) provides an apparent K_{inact} of 159 ± 21 nM and represents the inhibitor concentration required to produce a half maximal rate of inactivation. The first-order rate constant for inactivation was calculated to be 8.4 x 10^{-3} sec^{-1}. The half-time of inactivation at infinite inhibitor concentration is 1.38 ± 0.92 min and is more rapid than the values reported for 10β-propargylestr-4-ene-3,17-dione and 4-hydroxy-androstenedione (25).

Cell Culture Studies

The MCF-7 human cancer cell line has been utilized extensively as a model system for studying the regulation of breast cancer cell growth by steroids (26-28). Aromatase activity has been identified in these cell cultures (29) and comparative studies on several aromatase inhibitors have been performed (30). Evaluation of the 7α-substituted steroids

Scheme I



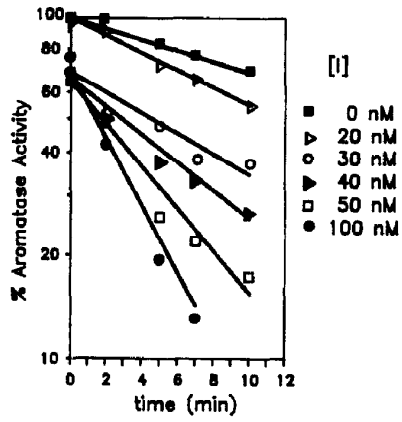


Figure 1. Inactivation of aromatase by 7α-APTADD with NADPH.

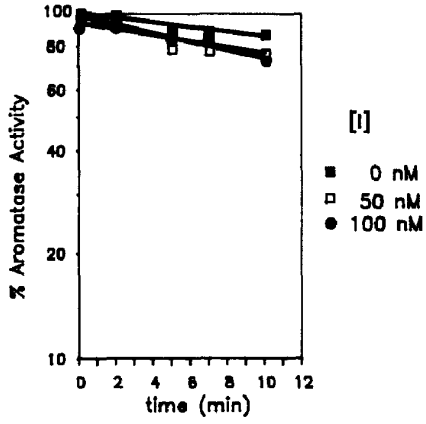


Figure 2. Inactivation of aromatase by 7α-APTADD without NADPH.

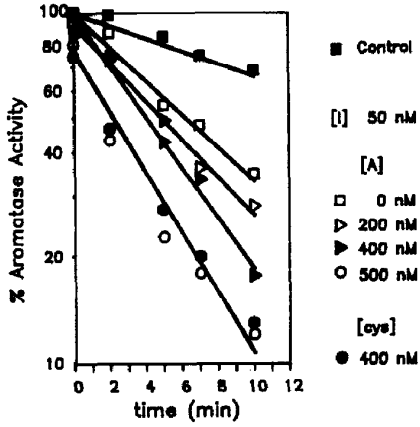


Figure 3. Protection studies with substrate or cysteine.

in cell culture systems and in vivo is critical for development of these compounds as potential therapeutic agents. 7α-APTA was examined in the MCF-7 cell culture system for its aromatase inhibitory activity, for its effects on estrogenic responses in these cells, and for any hormonal activities of 7α-APTA itself (31).

Aromatase activity in the MCF-7 cell culture system was measured using two radiotracer assays. The first assay examined the extent of conversion of [1,2,6,7-^{3}H]testosterone to [1,2,6,7-^{3}H]estradiol (29). The amount of estradiol formed in control cultures (no inhibitor) was found to be 23.73 pmol (± 7.10) per flask per 6 h or approximately 2.37 pmol per 10^6 cells per 6 h. 7α-APTA was examined at concentrations of 10 pM to 1 μM and effectively inhibited MCF-7 aromatase activity, providing a classical dose-response curve (Fig. 4) with an ED_{50} of 25.07 nM (± 7.71). Aromatase activity in MCF-7 cells was also determined by measuring the amount of ^3H_2O released from [1-^{3}H]androstenedione. Again, 7α-APTA effectively inhibited MCF-7 aromatase activity.

Potential hormonal effects of 7α-APTA in MCF-7 cells were investigated by examining this inhibitor for its ability to bind to the cytosolic estrogen receptor and its effect on progesterone receptor levels (30). 7α-APTA did not displace radiolabeled estradiol from these high affinity binding sites at concentrations up to 1 μM. In addition, 7α-APTA did not induce the production of progesterone receptors above control levels over the concentration range of 10 pM to 1 μM, while estradiol increased the levels of progesterone receptor in a dose-dependent fashion, with a maximum 2.4-fold increase (Fig. 5).

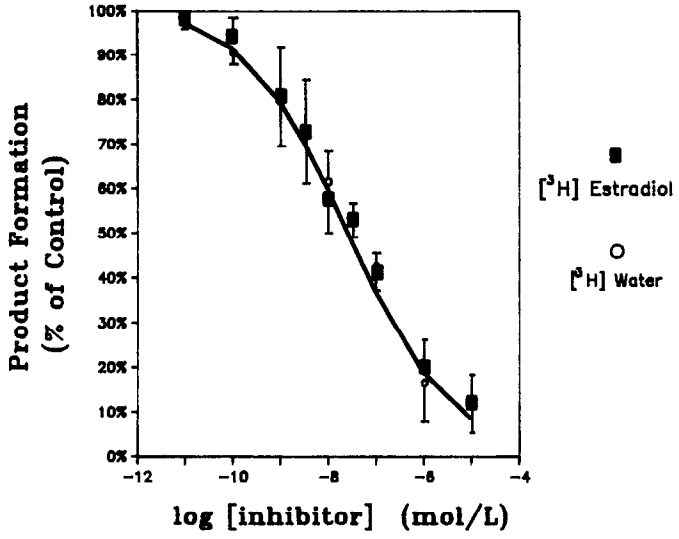


Figure 4. Inhibition of MCF-7 cell aromatase activity by 7α-APTA.

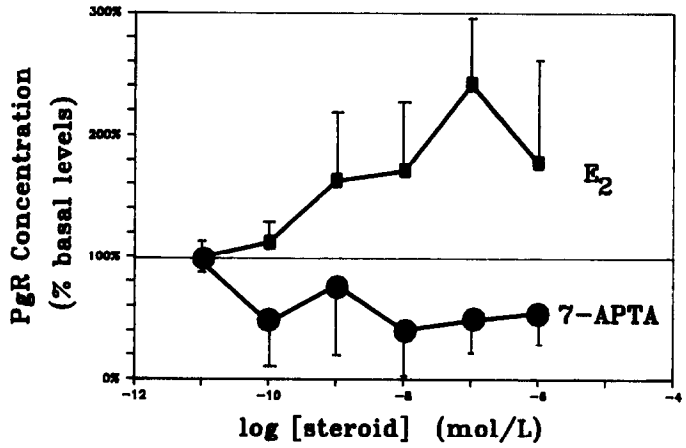


Figure 5. Effects of 7α-APTA and estradiol on levels of progesterone receptors in MCF-7 cells.

Tumored Animal Studies

The initial 7α-substituted C_{19} steroidal aromatase inhibitor examined in tumored animals was 7α-APTA. This competitive inhibitor was suspended in sesame oil and each rat was injected subcutaneously daily for 6 weeks at dosages of 25 or 50 mg/kg/day. The rats were weighed twice a week and the number and volume of the tumors present determined over a 6-week period. Rats in the control group received only sesame oil. The tumors of the control group grew steadily during the study, reaching an increase in total tumor volumes of approximately 550% of the original volumes (Fig. 6). On the other hand, the 7α-APTA treated groups demonstrated a reduction in tumor volumes during the first week. Furthermore, tumor volumes continued to decrease to less than 20% of the original volumes (80% reduction) during the treatment with 50 mg/kg/day. The group receiving 25 mg/kg/day responded with approximately a 50% reduction in tumor volume by the second week of treatment and maintained a 30-40% reduction of total tumor volumes throughout the rest of the 6-week study.

Since 7α-APTA was effective at a dose of 50 mg/kg body wt/day, experiments were performed to determine if this tumor reduction is due to inhibition of estrogen biosynthesis. Tumor-bearing rats were administered only 7α-APTA at 50 mg/kg/day for the first 3 weeks, followed by co-administration of 7α-APTA at 50 mg/kg/day and estradiol at 0.3 μg per kg per day for the last three weeks. Again, the tumors responded to 7α-APTA treatment with tumor reduction of 70% of the original volume during the first three weeks. Beginning at week 3, the co-administration of estradiol resulted in tumor growth (Fig. 6).

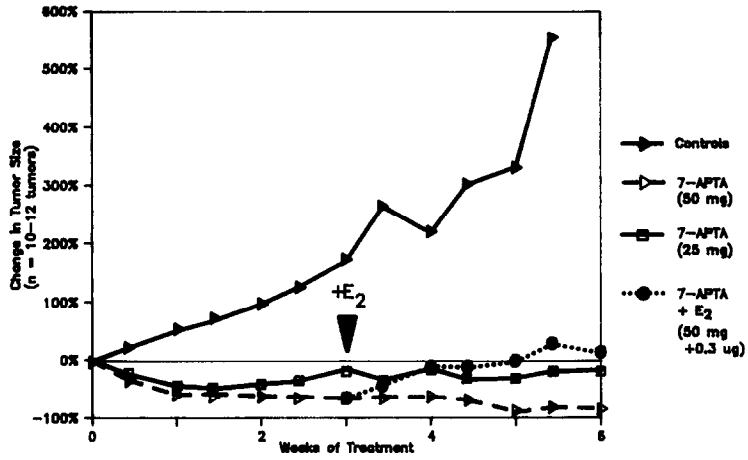


Figure 6. Effects of 7α-APTA on tumor growth.

DISCUSSION

Pharmacological manipulation of estrogen-dependent processes by lowering estrogen levels in vivo may prove an effective therapeutic strategy for treating disease states associated with high levels of estrogen. Several 7α-substituted androstenedione derivatives have demonstrated high affinity and potent inhibition of microsomal aromatase (6-10). 7α-(4'-Amino)phenylthio-4-androstene-3,17-dione (7α-APTA) is among the most potent competitive inhibitors (6), demonstrating an apparent K_i of 18 nM in microsomal enzyme preparations from human term placenta.

Enzyme-activated or enzyme-catalyzed inhibitors of aromatase could provide an excellent way of reducing estrogen biosynthesis. The use of these inhibitors would be advantageous due to their long duration of

action and high specificity. The reactive intermediate generated by enzyme catalysis forms a covalent bond with the enzyme at the active site, resulting in irreversible inhibition of the enzyme (14,15). The introduction of a C_1-C_2 double bond into androstenedione derivatives results in aromatase inactivation apparently by an enzyme-catalyzed mechanism (23). The combination of this structural modification with a 7α-(4'-amino)phenylthio moiety results in the compound 7α-(4'-amino)phenylthio-1,4-androstadiene-3,17-dione (7α-APTADD).

7α-APTADD is a potent inhibitor of aromatase with a K_I of 9.9 nM and produces a first-order inactivation of aromatase only in the presence of NADPH (11). Thus, inactivation is dependent on enzyme catalysis of the inhibitor. Androstenedione protected the enzyme from this inactivation process, suggesting that 7α-APTADD may be acting at or near the active site of aromatase. Cysteine had no effect on aromatase inactivation by 7α-APTADD. An evaluation of the inactivation kinetics demonstrates that this inhibitor produces a very rapid and efficient inactivation of aromatase with a minimum half-time of 1.38 min at saturating conditions. Thus, these studies support an enzyme-catalyzed mechanism of inhibition for 7α-APTADD in which the inhibitor initially binds with high affinity to aromatase and is then catalytically converted to a reactive intermediate. The enzyme subsequently reacts rapidly with the enzyme-activated inhibitor to form a covalent bond leading to irreversible inhibition.

The mechanism of aromatase inactivation following enzymatic catalysis by various steroids containing the 1,4-dien-3-one structural moiety remains unknown. The enhanced affinity of aromatase for

7α-APTADD may permit more detailed mechanistic studies to be performed. Furthermore, 7α-APTADD should be an effective medicinal agent for the treatment of estrogen-dependent tumors due to its high specificity and irreversible inhibition of aromatase.

Evaluation of 7α-substituted androstenediones as aromatase inhibitors in MCF-7 cells has provided further information on the efficacy of these agents as potential medicinal agents for the treatment of estrogen-dependent cancers. The biosynthesis of estrogens by aromatase present in MCF-7 cells can be quantitated either by measurement of the [^{3}H]estradiol product or by measurement of ^3H_2O released from [1-^{3}H]androstenedione. 7α-APTA inhibited this enzymatic activity in MCF-7 cells in culture in a dose-dependent fashion (Fig. 1), with an ED_{50} of 25.07 nM (± 7.71). This ED_{50} value is similar to the apparent K_i value of 18 nM from human placental microsomes (6).

The MCF-7 cell culture system is a valuable model for examining hormonal effects of natural and synthetic compounds. Estrogen receptors are present in these cells and estrogenic responses, particularly the induction of progesterone receptors following estrogen exposure, are observed. 7α-APTA was not able to displace tritiated estradiol from high-affinity binding sites on the estrogen receptor. Furthermore, 7α-APTA does not induce the production of progesterone receptors at concentrations up to 1 μM (Fig. 4). Thus, this aromatase inhibitor does not bind to cytosolic estrogen receptors or induce estrogenic responses. Additionally, these results suggest that 7α-APTA is not converted to an estrogenic metabolite in the cultures which would be capable of inducing progesterone receptors.

Finally, 7α-APTA was examined *in vivo* in the estrogen-dependent DMBA-induced mammary carcinoma rat model. Effective reduction of tumor volumes was observed with 7α-APTA at doses of 25 and 50 mg/kg/day (Fig. 6). Approximately 80% of tumors responded either completely or partially to 7α-APTA at the two doses examined. Co-administration of estradiol from week 3 to week 6 to rats receiving 50 mg/kg/day doses of 7α-APTA resulted in the reversal of tumor reduction in 10 of 12 tumors, resulting in 6 tumors having a greater volume than their original volumes at the end of the treatment period. Interestingly, 2 tumors that completely regressed during the first 3 weeks did not reappear upon estradiol co-administration.

Thus, 7α-APTA is an effective inhibitor of aromatase in intact MCF-7 cells and is effective in reducing tumor volumes in the estrogen-dependent DMBA-induced mammary carcinoma rat model. In addition, a new aromatase inhibitor, 7α-APTADD, has shown potent enzyme-catalyzed inactivation of aromatase *in vitro*. These studies encourage further development of this group, the 7α-substituted androgen analogs, as medicinal agents for the treatment of estrogen-dependent mammary carcinoma.

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