Aromatase Inhibition by an Enzyme-activated Irreversible Inhibitor in Human Carcinoma Cell Cultures

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ABSTRACT

7α-(4'-Amino)phenylthio-1,4-androstadiene-3,17-dione (7α-APTADD), a potent enzyme-activated irreversible inhibitor, was examined in two different human cell culture lines, MCF-7 human mammary carcinoma cells and JAr choriocarcinoma cells. Both the MCF-7 and JAr cell culture systems exhibit aromatase activity, and 7α-APTADD was evaluated for its aromatase-inhibitory activity, for its ability to inactivate the enzyme complex, and for the time course of recovery of enzymatic activity. This inhibitor produced a dose-dependent inhibition of aromatase activity in MCF-7 cells and in JAr cells, with EC50 values of 91 and 7.3 nm, respectively. Two other steroidal inhibitors, 7α-(4'-aminophenylthio-1,4-androstene-3,17-dione and 4-hydroxyandrostenedione, produced similar dose-response curves and EC50 values, while the nonsteroidal aminoglutethimide was less effective. Both cell culture systems exhibited prolonged inhibition of aromatase activity following exposure to 7α-APTADD, suggesting enzyme inactivation by this inhibitor. Thus, 7α-APTADD is an effective inhibitor of aromatase in MCF-7 mammary carcinoma cells and in JAr choriocarcinoma cells. These studies encourage further development of this group of medicinal agents for the treatment of estrogen-dependent mammary carcinoma.

INTRODUCTION

Inhibitors of aromatase, the cytochrome P-450 enzyme complex responsible for estrogen biosynthesis, may be useful in controlling reproductive processes and in treating estrogen-dependent disease states such as breast and endometrial cancer. These agents may be particularly effective in treating hormone-dependent breast cancer in postmenopausal patients, 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 (4-7).

7α-Substitution of androstenedione results in inhibitors of enhanced affinity for aromatase in vitro (8-14), with 7α- APTA3 being among the most potent competitive inhibitors produced to date (8). This agent effectively inhibited aromatase activity present in MCF-7 human mammary carcinoma cells in culture, exhibiting an EC50 of 25 nm (15). 7α-APTA has also demonstrated effectiveness in reducing 7,12-dimethylbenz[a]anthracene-induced, estrogen-dependent, mammary tumors in rats (16).

7α-APTADD is the most potent enzyme-activated irreversible inhibitor produced to date (13). In microsomal enzyme preparations from human term placenta, this compound exhibited an apparent Ki of 10 nm. 7α-APTADD produced inactivation of microsomal aromatase only in the presence of NADPH, with an apparent rate of inactivation of 8.4 x 10^3 s^-1. Evaluation of aromatase inhibition and inactivation by 7α-APTADD in cell culture systems will provide further information critical for development of these compounds as potential therapeutic agents.

The MCF-7 human mammary cancer cell line has been utilized extensively as a model system for studying the regulation of breast cancer cell growth by steroids (17-21). Aromatase activity has been demonstrated in these cell cultures (15, 22), and studies on several aromatase inhibitors have been performed (15, 23). The JAr choriocarcinoma cell line has high levels of aromatase and also has been utilized to evaluate aromatase inhibition (24-27). This report describes the evaluation of 7α-APTADD in the MCF-7 and JAr cell culture systems for its aromatase-inhibitory activity, for its ability to inactivate the enzyme complex, and for the time course of recovery of enzymatic activity.

MATERIALS AND METHODS

Steroids were obtained from Steraloids (Wilton, NH) and checked for purity by melting point and thin layer chromatography. [1-3H]-Androstene-3,17-dione was purchased from Du Pont-New England Nuclear (Boston, MA) and purity was checked by thin layer chromatography. MCF-7 human mammary carcinoma cells were obtained from the Ohio State University Cell Culture Service. JAr human choriocarcinoma cells were obtained from American Type Culture Collection (Rockville, MD). A modified Eagle's MEM supplemented with essential amino acids (1.5x), vitamins (1.5x), nonessential amino acids (2x), and L-glutamine (1x) was obtained in powdered form from Gibco (Long Island, NY). RPMI medium was also obtained in powdered form from Gibco. The sterilized liquid medium was prepared by the OSU Comprehensive Cancer Center Media Preparation Service by dissolving the powder in water containing sodium chloride (0.487 g/liter), pyruvic acid (0.11 g/liter), sodium bicarbonate (1.5 g/liter), and phenol red (0.01%), and the pH was 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 (28). Tissue culture flasks and supplies were obtained from Corning Glass Works (Corning, NY). Biochemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Radioactive samples were detected with a Beckman LS 6800 scintillation counter, using Formula 963 (Du Pont-New England Nuclear) as the counting solution. EC50 values were calculated by a nonlinear regression analysis using the Marquardt method (SAS Institute, Cary, NC).

Inhibition of MCF-7 Aromatase Activity. MCF-7 mammary carcinoma cells were grown in 150-cm² plastic flasks at 37°C in a modified Eagle's MEM (20 ml) containing 10% fetal calf serum. The aromatase inhibitors were added to cultures that were 90% confluent (approximately 1 to 2 x 10⁷ cells), at concentrations of 10 µm to 1 µm in 10 µl ethanol. For all cell culture studies, experiments were carried out using triplicate flasks. Aromatase activity was determined by measuring the conversion of [1-3H]-Androstenedione to H₂O and unlabeled estrone (15). For aromatase inhibition, [1-3H]-Androstenedione (30 nm, 2 µCi) was dissolved in 10 µl 95% ethanol and added to the cultures. At the same time, varying concentrations of aromatase inhibitor (10⁻¹² to 10⁻⁷ M) were dissolved in 10 µl 95% ethanol and added to the cultures. Control cultures received [3H]-Androstenedione, unlabeled estradiol, and no inhibitor (ethanol only).

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3 The abbreviations used are: 7α-APTA, 7α-(4'-aminophenylthio-1,4-androstene-3,17-dione; 7α-APTADD, 7α-(4'-aminophenylthio-1,4-androstadiene-3,17-dione; MEM, minimum essential medium; AG, aminoglutethimide; 4-OHA, 4-hydroxyandrostenedione.
Blank samples contained [3H]androstenedione and unlabeled estradiol in medium only (no cells). At 24 h the flasks were removed from the incubator. The media were extracted 3 times with ethyl acetate (20 ml each time). An aliquot (1.0 ml) of the aqueous medium layer was added to Formula 963 (4.0 ml) and the gel was analyzed by liquid scintillation counting to determine the amount of radioactivity. DNA content was measured by a modified diphenylamine assay (29). The percentage of inhibition was determined by dividing the total amount of $^3$H$_2$O formed in the particular inhibitor sample by the amount of $^3$H$_2$O formed in the uninhibited (control) samples.

**Aromatase Inactivation in MCF-7 Cells.** MCF-7 cell cultures (approximately 90% confluent) in 75-cm$^2$ plastic flasks containing modified Eagle’s MEM (5 ml) were incubated with 7α-APTADD (100 nM, dissolved in 5 μl 95% ethanol) for 24 h. The medium was then changed, cells were washed with phosphate-buffered saline, fresh medium was added, and [1-3$^3$H]androstenedione (50 nM, 2.5 μCi, in 5 μl ethanol) was added for 2-h incubations at 0, 1, 2, 3, 4, and 5 days. The medium was then transferred to 35-ml centrifuge tubes and frozen. Control wells were treated in the identical fashion without added inhibitor. The medium was extracted as described earlier and the amount of $^3$H$_2$O formed was determined. DNA determinations were performed on the cells in the culture plates. Samples were analyzed in quadruplicate at each time and inhibitor concentration.

**Inhibition of JAr Aromatase Activity.** JAr cells were grown in 75-cm$^2$ plastic flasks at 37°C, in RPMI medium (10 ml) containing 10% fetal calf serum and gentamycin (20 mg/l). For aromatase activity, JAr cells were divided into 9.4-cm$^2$ wells in RPMI medium (2 ml). When cultures reached 90% confluency (approximately 3 to 4 x 10$^6$ cells), medium was changed and varying concentrations of aromatase inhibitors (10$^{-12}$ to 10$^{-4}$ M, in 5 μl 95% ethanol) were added. Experiments were carried out in triplicate. Aromatase activity was determined by measuring the conversion of [1-3$^3$H]androstenedione to $^3$H$_2$O and unlabeled estrone. For aromatase inhibition, [1-3$^3$H]androstenedione (50 nM, 2.5 μCi, in 5 μl ethanol) was added to the cultures at the same time as the inhibitor. Control cultures received [1-3$^3$H]androstenedione, 95% ethanol, and no inhibitor. Blank samples contained [1-3$^3$H]androstenedione in medium only (no cells). After 2 h the plates were removed from the incubator and the medium was transferred into 35-ml centrifuge tubes. Chloroform (10 ml) was added to the medium and the samples were vortexed, incubated at room temperature for 30 min, and then centrifuged at 500 x g for 10 min. Three aliquots (500 μl) of the supernatant were transferred into 12 x 75-mm culture tubes, and 1% dextran-coated charcoal solution was added (500 μl). After vortexing, the tubes were incubated at 4°C for 15 min and then centrifuged for 10 min at 2600 x g. The supernatant was mixed with Formula 963 cocktail and analyzed by liquid scintillation counting. DNA content of the cultures was determined by the diphenylamine assay (29). The percentage of inhibition was determined by dividing the total amount of estradiol formed (pmol/μg DNA) in the inhibited sample by the amount of estradiol formed in the uninhibited (control) samples.

**Aromatase Inactivation in JAr Cells.** JAr cell cultures (approximately 90% confluent) in 9.4-cm$^2$ wells containing RPMI medium (2 ml) were incubated with 7α-APTADD (5 or 50 nM, dissolved in 5 μl 95% ethanol) or with 7α-APTA (50 nM, dissolved in 5 μl 95% ethanol) for 2 h. The medium was then changed, cells were washed with phosphate-buffered saline, fresh medium was added, and [1-3$^3$H]androstenedione (50 nM, 2.5 μCi, in 5 μl ethanol) was added for a 20-min incubation at 0, 6, 12, 24, 36, and 48 h. The medium was then transferred to 35-ml centrifuge tubes and frozen. Control wells were treated in the identical fashion without added inhibitor. The medium was extracted as described earlier and the amount of $^3$H$_2$O formed was determined. DNA determinations were performed on the cells in the culture plates. Samples were analyzed in quadruplicate at each time and inhibitor concentration.

**RESULTS**

Inhibition of MCF-7 Aromatase Activity. Inhibition of aromatase activity present in MCF-7 human mammary carcinoma cell culture was determined by measuring the conversion of [1-3$^3$H]androstenedione to $^3$H$_2$O and unlabeled estrone. This radiometric assay method for measuring aromatase activity was previously validated with a production isolation assay method in the MCF-7 cell culture system (15). The level of aromatase activity present in these MCF-7 cell cultures was 0.975 ± 0.36 pmol product formed/10$^6$ cells/h or approximately 53 pmol/flask in 24 h.

The enzyme-activated irreversible inhibitor 7α-APTADD (1, Fig. 1) was evaluated for aromatase inhibition and compared to 7α-APTA (2, Fig. 1), 4-OHA (3, Fig. 1), and AG (4, Fig. 1). The three steroidal inhibitors produced similar dose-response curves (Fig. 2), with EC$_{50}$ values ranging from 25 to 91 nM, while the nonsteroidal AG was less effective, with an EC$_{50}$ value of 1.79 μM (Table 1).

Aromatase Inactivation in MCF-7 Cells. 7α-APTADD was also evaluated for its ability to produce long term inhibition of aromatase in MCF-7 cells. The inhibitor, at a concentration of 100 nM, was incubated with MCF-7 cells for a 24-h period. The medium was then removed from the MCF-7 cell cultures, the

**Table 1** EC$_{50}$ values for aromatase inhibitors in cell cultures

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>MCF-7 cells</th>
<th>JAr cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>7α-APTADD</td>
<td>91.4</td>
<td>7.3</td>
</tr>
<tr>
<td>7α-APTA</td>
<td>25.1</td>
<td>105.0</td>
</tr>
<tr>
<td>4-OHA</td>
<td>46.4</td>
<td>3.5</td>
</tr>
<tr>
<td>AG</td>
<td>1,791.7</td>
<td>13,129.0</td>
</tr>
</tbody>
</table>
cells were washed with phosphate-buffered saline, and fresh medium was added. The resultant aromatase activity was monitored for the next 5 days by radiometric assays performed over 2-h incubation periods. The results were compared to control flasks, which received no inhibitor, with the data presented as percentage of control aromatase activity (Fig. 3). Immediately after removal of the medium containing 7α-APTADD and washing of the cells, the aromatase activity in inhibitor-treated cultures was approximately 50% of the activity in control cultures. The aromatase activity in inhibitor-treated cultures gradually increased over the next 3 days, to approximately 80% of the activity of control cultures. The aromatase activity in the inhibitor-treated MCF-7 cells then stabilized at approximately 80% of control cultures from day 3 to day 5.

Inhibition of JAr Aromatase Activity. Aromatase inhibition was also evaluated in choriocarcinoma cell cultures. The JAr choriocarcinoma cell line was selected for the studies because these cells have high levels of aromatase (24–26) and have been utilized to evaluate aromatase inhibition (27). Again, aromatase activity was determined by measuring the conversion of [1-3H]-androstenedione to \( \text{H}_2\text{O} \) and unlabelled estrone. The level of aromatase activity present in these JAr cell cultures was 6.31 ± 2.17 pmol formed/10⁶ cells/h, or approximately 44.15 pmol/well in 2 h. The three steroid inhibitors, 7α-APTADD, 7α-APTA, and 4-OHA, produced similar dose-response curves (Fig. 4), with \( EC_{50} \) values ranging from 3 to 105 nM, while the nonsteroidal AG was much less effective, with an \( EC_{50} \) value of 13.1 µM (Table 1).

Aromatase Inactivation in JAr Cells. 7α-APTADD was also evaluated for its ability to produce long-term inhibition of aromatase in JAr cells. This enzyme-activated irreversible inhibitor was examined at concentrations of 5 or 50 nM and was incubated with JAr cells for a 2-h period. The medium was then removed from the JAr cell cultures, the cells were washed with phosphate-buffered saline, and fresh medium was added. The resultant aromatase activity was monitored for the next 2 days by radiometric assays performed over 20-min incubation periods. The competitive inhibitor 7α-APTA was examined under the same conditions at an inhibitor concentration of 50 nM. The results were compared to control flasks, which received no inhibitor, with the data presented as percentage of control aromatase activity (Fig. 5). Immediately after removal of the medium containing inhibitor and washing of the cells, the aromatase activity in all the inhibitor-treated cultures was approximately 10–20% of the activity in control cultures. The aromatase activity in cultures treated with 7α-APTADD at 5 nM increased rapidly over the next 12 h and returned to control levels by 24 h. On the other hand, the aromatase activity in cultures treated with 7α-APTADD at 50 nM increased only gradually over the next 36 h to control levels. Finally, the aromatase activity in cultures treated with 7α-APTA at 50 nM increased rapidly over the next 12 h, and the results were similar to those for the low concentration of 7α-APTADD.

**DISCUSSION**

7α-Substituted androstenediones are very effective aromatase inhibitors when examined in human placental microsomes (8–14). Efficacy of the potent competitive inhibitor 7α-APTA has been demonstrated in MCF-7 cells (15) and in treatment of estrogen-dependent mammary tumors in rats (16). Furthermore, 7α-ATPA did not result in estrogen responses in the MCF-7 cell line, as evidenced by no induction of progesterone receptor levels and no effect on cell growth (15). Evaluation of new aromatase inhibitors in cell culture systems will provide data on the effectiveness of these agents in intact cells.
7α-APTADD, a potent enzyme-activated irreversible inhibitor, was examined initially in MCF-7 human mammary carcinoma cell cultures. 7α-APTADD inhibited aromatase activity in MCF-7 cells in a dose-dependent fashion (Fig. 2), with an EC$_{50}$ of 91 nM. This EC$_{50}$ value is slightly higher than the apparent $K_i$ value of 10 nM from human placental microsomes (13). Three other aromatase inhibitors, 7α-APTA, 4-OHA, and AG, were also examined in MCF-7 cells. 7α-APTA and 4-OHA gave EC$_{50}$ values of 25 and 46 nM, similar to that of 7α-APTADD (Table 1). The EC$_{50}$ for AG was significantly higher, at 1,791 μM. These EC$_{50}$ results for 4-OHA and AG in MCF-7 cells are higher than those reported previously of 0.1 and 400 nM, respectively (23). Culture heterogeneity, varying culture conditions employed, and genetic instability of continuously cultured cancer cells may explain these differences in EC$_{50}$ values from various laboratories (30–32). Thus, such differences illustrate the importance of evaluating and comparing relative potencies of enzyme inhibitors in experiments performed under identical cell culture conditions.

Enzyme inactivation was demonstrated with 7α-APTADD in placental microsomal preparations only under catalytically active enzymatic conditions (13). MCF-7 cell cultures were utilized to determine if such inactivation, and thus long-acting enzyme inhibition, could be observed. After a 24-h exposure of MCF-7 cells to 7α-APTADD, inhibitor-treated cultures exhibited approximately 50% of the aromatase activity of control cultures. The aromatase activity in inhibitor-treated cultures gradually increased over the next 3 days, to approximately 80% of the activity of control cultures, and stabilized at that level. Thus, the MCF-7 cells exposed to 7α-APTADD for 24 h needed an additional 72 h (3 days) in culture to order to return aromatase activity to near normal levels.

The efficacies of 7α-APTADD and 7α-APTA to inhibit aromatase activity in JAr trophoblastic choriocarcinoma cells were also evaluated. This cell line was selected for evaluation of the inhibitors because of high levels of aromatase present in the JAr cells, which permit the use of smaller cell numbers, less medium, and shorter incubation times in measuring enzymatic activity. 7α-APTADD and 7α-APTA inhibited aromatase activity in JAr cells in dose-dependent fashions (Fig. 4), with EC$_{50}$ values of 7.3 and 105 nM, respectively. 4-OHA and AG were also examined in these cultures. 4-OHA had an EC$_{50}$ value similar to that of 7α-APTADD (Table 1), while that for AG was much higher at 13.1 μM. Thus, high levels of aromatase were detected and enzyme inhibition was observed in the JAr cell culture system. Furthermore, the assay methods employing small cell numbers and shorter incubation times enable more rapid and quantitative determinations of inhibitory activity when examining potential aromatase inhibitors. Interestingly, 7α-APTA, a competitive inhibitor, was approximately 10 times less active than 7α-APTADD, an enzyme-activated irreversible inhibitor, in the JAr cells. Additionally, the difference in EC$_{50}$ values for 7α-substituted C$_{19}$ steroids obtained in JAr cells from those values in MCF-7 cells suggest that cellular environments of membrane-bound aromatase may be different between these cells derived from two different tissue sources.

Finally, aromatase inactivation by 7α-APTADD was also evaluated in JAr choriocarcinoma cell cultures at two different inhibitor concentrations. As a control, identical studies were performed with the potent competitive inhibitor 7α-APTA. After a 2-h exposure of MCF-7 cells to 7α-APTADD, inhibitor-treated cultures exhibited approximately 10–20% of the aromatase activity in control cultures. The aromatase activity in cultures treated with 7α-APTADD at 50 nM increased only gradually over the next 36 h to control levels. Thus, the JAr cells exposed to 7α-APTADD for 2 h needed an additional 36 h in culture in order to return aromatase activity to normal levels. The results from cultures treated with 7α-APTA at 50 nM demonstrated a rapid increase in aromatase activity over 12 h and a return to control levels by 24 h. Therefore, a prolonged suppression of aromatase activity in JAr cells treated with 7α-APTADD suggests that enzyme inactivation is occurring in cultures. Additionally, the difference in the results between 7α-APTADD and 7α-APTA, two steroids with very similar structures and chemical properties, indicates that the prolonged suppression produced by 7α-APTADD is not due to clearance of the compound from the cultures. At lower concentrations of 7α-APTADD (5 nM), aromatase activity increased rapidly over the next 12 h; these results were similar to those of 7α-APTA.

Thus, the aromatase inhibitor 7α-APTADD is an effective inhibitor of aromatase in intact MCF-7 mammary carcinoma cells. This inhibition is similar to that observed for two other potent steroidal agents, 7α-APTA and 4-OHA. Additionally, both 7α-APTADD and 7α-APTA inhibit aromatase activity in JAr choriocarcinoma cells, with 7α-APTADD demonstrating greater efficacy. Furthermore, 7α-APTADD demonstrates prolonged suppression of aromatase activity in both cell culture systems, suggesting enzyme-mediated inactivation of aromatase. These studies encourage further development of this group of medicinal agents for the treatment of estrogen-dependent mammary carcinoma.

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