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BIOCHEMICAL AND PHARMACOLOGICAL DEVELOPMENT OF STEROIDAL INHIBITORS OF AROMATASE

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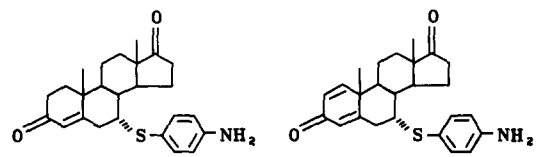
Summary—Androstenedione analogs containing 7α-substituents have proven to be potent inhibitors of aromatase both *in vitro* and *in vivo*. Several of these agents have exhibited higher affinity for the enzyme complex than the substrate. In order to examine further the interaction(s) of 7-substituted steroids with aromatase, biochemical and pharmacological studies were performed on 7α-thiosubstituted androstenediones and 7-substituted 4,6-androstadiene-3,17-diones. Potent inhibition of aromatase activity in human placental microsomes has been observed with several new 7α-thiosubstituted androstenediones. 7-Benzyl- and 7-phenethyl-4,6-androstadiene-3,17-diones effectively inhibited microsomal aromatase, with apparent K_is ranging from 61 to 174 nM. On the other hand, 7-phenyl-4,6-androstadiene-3,17-dione exhibited poor activity, with an apparent K_i of 1.42 μM. Similar inhibitory activity was observed with reconstituted, purified cytochrome $P450_{Arom}$ preparations. Additionally, these agents were evaluated for inhibition of aromatase activity in two human carcinoma cell lines, the MCF-7 human mammary cancer line and the JAr choriocarcinoma line. The 7α-thiosubstituted androstenediones and 7-substituted 4,6-androstadiene-3,17-diones produced dose-dependent inhibitions of aromatase activity in the cell cultures. The most effective inhibitors were the 7α-substituted androstenediones, with EC_{50} values ranging from 7.3 to 105 nM. Finally, the JAr cell culture system exhibited prolonged inhibition of aromatase activity following exposure to 7α-APTADD, suggesting enzyme inactivation by this inhibitor. Thus, these agents are effective aromatase inhibitors, and the results encourage further development of this group of medicinal agents for the treatment of estrogen-dependent mammary carcinoma.

INTRODUCTION

Several 7α-thiosubstituted derivatives of androstenedione have demonstrated enhanced affinity for aromatase and produced very effective inhibition of aromatase activity present in human placental microsomes [1–7]. This group of inhibitors includes competitive, affinity, photoaffinity, and enzyme-activated irreversible inhibitors. 7α-(4′-Amino)phenylthio-4-androstene-3,17-dione (7α-APTA, **1**, Fig. 1), is one of the most potent competitive inhibitors, with an apparent K_i of 18 nM [1]. This inhibitor has also demonstrated effectiveness in inhibiting aromatase in cell cultures [7, 8] and in treating hormone-dependent rat mammary tumors [7, 9]. Affinity labeling derivatives of 7α-APTA produced inactivation of aromatase, with a ^{14}C-analog demonstrating covalent binding to aromatase [2, 3].

Androstenedione derivatives with extended linear conjugation in ring A and/or B produced effective inhibition of aromatase [10]. Furthermore, the introduction of an additional double bond in the A-ring resulted in inhibitors that inactivated aromatase by an enzyme-catalyzed process [11–13]. Introduction of a 7α-substituent on 1,4-androstadiene-3,17-dione yielded a potent mechanism-based irreversible inhibitor of aromatase, 7α-(4′-amino)phenylthio-1,4-androstadiene-3,17-dione (7α-APTADD, **2**, Fig. 1). This compound exhibited an apparent K_i of 9.9 nM and has a half-time of inactivation (T_{50})



7α-APTA 7α-APTADD

Fig. 1. Aromatase inhibitors—7α-ATPA and 7α-ATPADD.

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of 1.38 min, the most rapid rate of inactivation of aromatase reported to date [6].

The introduction of substituents at C_7 of 4,6-androstadiene-3,17-dione may lead to enhanced affinity of these analogs for the aromatase complex. In addition, replacement of the carbon–sulfur bond with a carbon–carbon bond would yield analogs with similar lipophilic character and eliminate potential metabolic oxidation of the thioether linkage. Compounds with both features (extended linear conjugation in ring A and/or B and a carbon–carbon bond at C_7) have recently been prepared and exhibit good aromatase inhibition [14].

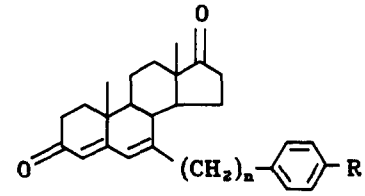
Current research activities by our laboratory have focused on the design of new 7-substituted C_{19} steroids for further examination of the structural requirements of the active site of aromatase and development of more effective medicinal agents. This manuscript summarizes recent biochemical and pharmacological evaluations of potent 7α-substituted androstenediones and 7-substituted 4,6-androstadiene-3,17-diones. Investigations have included enzyme inhibition studies with human placental microsomal preparations and with reconstituted, purified cytochrome $P450_{Arom}$ preparations. Additionally, these agents have also been studied for aromatase inhibition in two human carcinoma cell lines—the MCF-7 human mammary cancer line and the JAr choriocarcinoma line.

BIOCHEMISTRY

Studies with placental microsomes

Several 7-substituted 4,6-androstadiene-3,17-diones were evaluated *in vitro* by enzyme kinetic studies using human placental microsomes. These compounds included 7-phenyl-, 7-benzyl-, and 7-phenethyl-4,6-androstadiene-3,17-diones, compounds **3–9**, as shown in Table 1 [14]. Aromatase activity in human placental microsomes was assayed by the radiometric method in which the tritium in [1β-^{3}H]4-androstene-3,17-dione was transferred into water during aromatization. The amount of ^3H_2O released was used as an index of estrogen formation. All the inhibitors were evaluated at concentrations ranging from 0 to 600 nM in initial velocity studies performed under limiting enzyme concentrations. The results of the studies were plotted in a typical Lineweaver–Burk or double-reciprocal plot as 1/velocity vs 1/substrate. The apparent K_i of the inhibitor is an index of the affinity of the enzyme for the inhibitor and is determined by a weighted regression analysis computer program [15]. The apparent K_is for 7-substituted 4,6-androstadiene-3,17-diones **3–9** ranged from 61 nM to 1.42 μM (Table 1); the apparent K_m for the substrate androstenedione was 51 nM. Each inhibitor demonstrated competitive inhibition, as determined from the Lineweaver–Burk plots and V_{max} intercepts. Thus, the 7-benzyl and 7-phenethyl analogs were effective aromatase inhibitors while the 7-phenyl compound was a poor inhibitor. The low inhibitory activity of this latter derivative may be that the 7-phenyl substituent can only adopt a pseudo-β position relative to the planar steroid nucleus, whereas the 7-benzyl and 7-phenethyl groups of 4,6-androstadiene-3,17-diones have increased structural flexibility and can protrude in the 7α pocket.

Table 1. Aromatase inhibition by various 7-substituted 4,6-androstadienediones

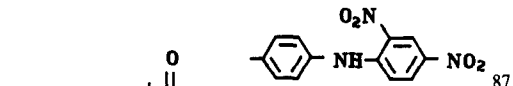
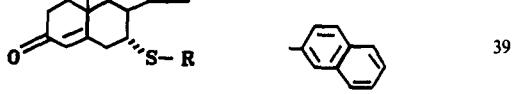
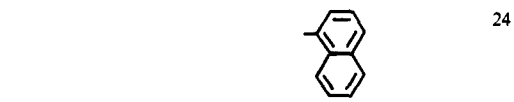


Compound	n	R	K_i (nM)	Inhibition
3 7-phenyl-ADD	0	—H	1424	Competitive
4 7-benzyl-ADD	1	—H	61	Competitive
5 7-phenethyl-ADD	2	—H	174	Competitive
6 7-nitrobenzyl-ADD	1	—NO_2	94	Competitive
7 7-aminobenzyl-ADD	1	—NH_2	88	Competitive
8 7-nitrophenethyl-ADD	2	—NO_2	95	Competitive
9 7-aminophenethyl-ADD	2	—NH_2	88	Competitive

K_m for androstenedione = 51 nM (SE = 9 nM).

Numerous effective 7α- and 7-substituted steroidal aromatase inhibitors have phenyl substitutents at the 7-position. Incorporation of other aryl moieties, such as fluorescent aromatic groups, would provide potentially new aromatase inhibitors with unique characteristics. A dinitrophenyl derivative of 7α-APTA (**10**) and two 7α-naphthylthio derivatives of androstenedione (**11** and **12**) were prepared in high yields and demonstrated good to excellent inhibition of placental microsomal aromatase, as shown in Table 2 [16]. Compounds **11** and **12**, the naphthylthio derivatives, exhibit high affinity for aromatase, as demonstrated by the excellent apparent K_is of 24.1 and 38.9 nM. These results indicated that 7α-substituents with bulky, planar functionalities can interact effectively at the active site of aromatase. Additionally, as evidenced by the higher K_i and lower affinity of compound **10**, modifications that extend the

Table 2. Fluorescent 7α-substituted aromatase inhibitors

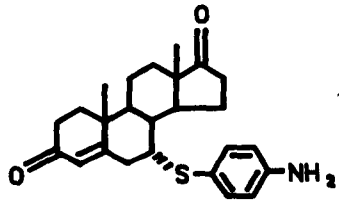
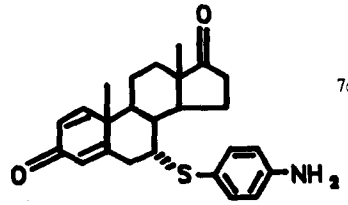
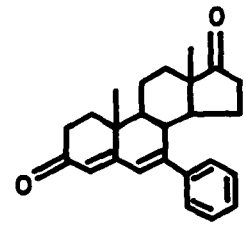
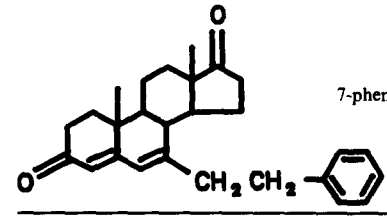
R	K_i(nM)
	87
	39
	24

7α-substituents away from the steroid nucleus lead to a decrease in affinity for the enzyme complex.

Studies with purified aromatase protein

Evaluation of the enzyme kinetics of 7α- and 7-substituted steroidal aromatase inhibitors with reconstituted preparations containing purified cytochrome $P450_{Arom}$ protein has recently been initiated in our laboratories. The aromatase protein (cytochrome $P450_{Arom}$) was purified following the procedures of Kellis and Vickery [17]. Briefly, the enzyme was extracted with sodium cholate, fractionated by ammonium sulfate precipitation, and subjected to column chromatography in the presence of substrate androstenedione and the nonionic detergent, Nonidet NP-40. This procedure yielded a highly purified and active cytochrome $P450_{Arom}$, with an overall protein yield of 5% and a specific content of 13.0 nmol of cytochrome $P450$ per mg of protein.

Table 3. Enzyme kinetics of 7-substituted steroidal aromatase inhibitors. Placental microsomes vs purified cytochrome $P450_{Arom}$

		Microsomes	Purified
Apparent K_m	Androstenedione	52 nM	82 nM
Apparent V_{max}	Androstenedione	0.23 nmol/mg·min	1.90 nmol/mg·min
Apparent K_i			
	7α-APTA	18 nM	11 nM
	7α-APTADD	10 nM	17 nM
	7-phenyl-ADD	1424 nM	846 nM
	7-phenethyl-ADD	174 nM	136 nM

Reconstitution of this cytochrome $P450_{Arom}$ with NADPH-cytochrome $P450$ reductase and phospholipid resulted in complete conversion of [1β-^{3}H]androstenedione to ^3H_2O, exhibiting an apparent K_m of 82 nM. The aromatase inhibitors 7α-APTA, 7α-APTADD, and several 7-substituted 4,6-androstadiene-3,17-diones were evaluated under initial velocity conditions in this reconstituted aromatase system (Table 3). The apparent K_is for these inhibitors varied from 10 to 850 nM. In general, the affinities of the various steroidal inhibitors examined with the purified protein more closely approximate the affinity of the substrate androstenedione. Inhibitors with high affinity in the microsomal preparations (e.g. 7α-APTA and 7α-APTADD) exhibit similar affinity with the purified protein. On the other hand, inhibitors with weaker affinity in the microsomal preparations (e.g. 7-phenyl-4,6-androstadiene-3,17-dione) exhibit increased affinity with the purified protein. These results indicate that specificity of the active site of aromatase is diminished upon removal of the cytochrome $P450_{Arom}$ protein from the native membrane environment.

CELL CULTURE INVESTIGATIONS

MCF-7 human mammary carcinoma

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 [18–20]. Aromatase activity has been demonstrated in these cell cultures [8, 21] and studies on several aromatase inhibitors have been performed [8, 22]. Inhibition of aromatase activity present in MCF-7 human mammary carcinoma cell culture was determined by measuring the conversion of [1β-^{3}H]androstenedione to ^3H_2O and unlabeled estrone. The level of aromatase activity present in these MCF-7 cell cultures was 0.975 ($\pm$0.36) pmol product formed per 10^6 cells per h, or approximately 53 pmol per T-150 flask in 24 h.

The enzyme-activated irreversible inhibitor 7α-APTADD was evaluated for aromatase inhibition and compared to 7α-APTA, 4-hydroxyandrostenedione (4-OHA), and aminoglutethimide (AG). The three steroidal inhibitors produced similar dose–response curves (Fig. 2), with EC_{50}s ranging from 25 to 91 nM, while the nonsteroidal AG was less effecitve with an EC_{50} value of 1.79 μM (Table 4). Thus, these investigations of aromatase inhibitors in the MCF-7 cell culture system provide data on the effectiveness of these agents in intact cells.

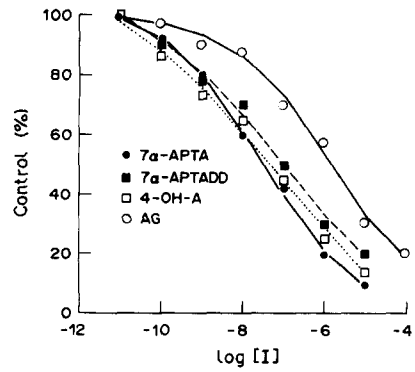


Fig. 2. Inhibition of aromatase activity in MCF-7 cell cultures. MCF-7 cells in 150 cm^2 flasks were incubated for 24 h at 37°C with radiolabeled substrate (30 nM) and 7α-APTADD [■], 7α-APTA [●], 4-OHA [□], or AG [○] at concentrations ranging from 10 pM to 100 μM. Aromatase activity was determined by measuring the amount of ^3H_2O released. Each point represents the average of three determinations, with variation of less than 15%. The value for 100% estradiol formation in control cultures (no inhibitor) was 0.975 ($\pm$0.36) pmol product formed per 10^6 cells per h, or 53 pmol per flask in 24 h.

JAr trophoblast choriocarcinoma

The efficacies of 7α-APTADD and 7α-APTA to inhibit aromatase activity in JAr trophoblastic choricarcinoma cells were also evaluated. This cell line has high levels of aromatase activity in the cells [23–25] and has been used in evaluation of an aromatase inhibitor [26]. In these studies, smaller numbers of cells, less media, and shorter incubation times in measuring enzymatic activity are used. Aromatase activity was determined by measuring the conversion of [1β-^{3}H]androstenedione to ^3H_2O and unlabeled estrone. The level of aromatase activity present in these JAr cell cultures was 6.31 ($\pm$2.17) pmol product formed per 10^6 cells per h, or approximately 44.15 pmol per 9.2 cm^2

Table 4. EC_{50}s of aromatase inhibitors in human carcinoma cell cultures

MCF-7 human mammary carcinoma cell cultures	
7α-APTA	25 nM
7α-APTADD	91 nM
4-OH-A	46 nM
Aminoglutethimide	1792 nM
JAr human trophoblast carcinoma cell cultures	
7α-APTA	105 nM
7α-APTADD	7 nM
4-OH-A	4 nM
Aminoglutethimide	13,129 nM
7-Benzyl-ADD	600 nM
7-Phenethyl-ADD	660 nM
7-Nitrobenzyl-ADD	1237 nM
7-Aminobenzyl-ADD	490 nM
7-Nitrophenethyl-ADD	4500 nM
7-Aminophenethyl-ADD	1246 nM

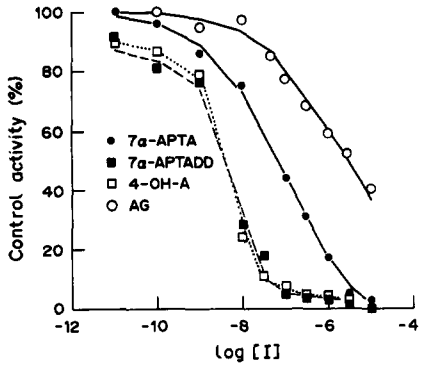


Fig. 3. Inhibition of aromatase activity in JAr cell cultures. JAr cells in 9.4 cm^2 wells were incubated for 2 h at 37°C with radiolabeled substrate (30 nM) and 7α-APTADD [■], 7α-APTA [●], 4-OHA [□], or AG [○] at concentrations ranging from 10 pM to 10 μM. Aromatase activity was determined by measuring the amount of ^3H_2O released. Each point represents the average of three determinations, with variation of less than 10%. The value for 100% estradiol formation in control cultures (no inhibitor) was 6.31 (±2.17) pmol product formed per 10^6 cells per h, or 44.15 pmol per well in 2 h.

well in 2 h. 7α-APTADD and 7α-APTA inhibited aromatase activity in JAr cells in dose-dependent fashions (Fig. 3), with EC_{50}'s of 7.3 and 105 nM, respectively (Table 4). 4-OHA and AG were also examined in these cultures. 4-OHA had a similar EC_{50} value to that of 7α-APTADD (3.5 nM), while that for AG was greatly higher at 13.1 μM. Several 7-substituted 4,6-androstadiene-3,17-diones were also evaluated in these cells and exhibited EC_{50}s ranging from 490 nM to 4.5 μM (Table 4).

Thus, high levels of aromatase were detected and enzyme inhibition was observed in the JAr cell culture system. Furthermore, the assay methods employing smaller cell numbers and shorter incubation times enable more rapid and quantitative determinations of inhibitory activity when examining potential aromatase inhibitors. On the other hand, 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.

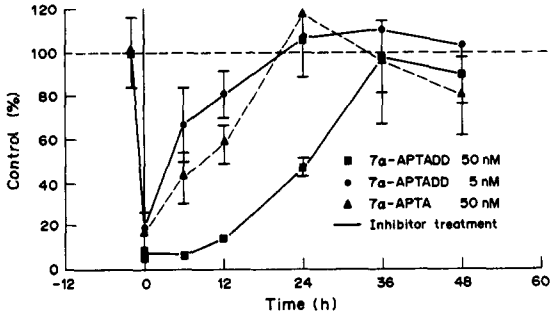


Fig. 4. Inactivation of aromatase activity in JAr cell cultures. JAr cells in 9.4 cm^2 wells were incubated for 20 min at 37°C with 7α-APTADD AT 5 nM [●], 7α-APTADD at 50 nM [■], or 7α-APTADD at 50 nM [▲]. The media was removed, cells washed, and fresh media added. Aromatase activity was measured at 0, 6, 12, 24, 36 and 48 h using the radiometric assay. Each point represents the average of 4 determinations.

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 a concentration of 50 nM and was incubated with JAr cells for a 2-h period. The media was then removed from the JAr cell cultures, the cells washed with phosphate-buffered saline, and fresh media 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 and serves as a competitive inhibition control. The results were compared to control flasks which received no inhibitor, with the data presented as % control aromatase activity (Fig. 4). Immediately after removing the media 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 50 nM increased only gradually over the next 36 h to control levels. On the other hand, the aromatase activity in cultures treated with 7α-APTA at 50 nM increased rapidly over the next 12 h and returned to control levels by 24 h. 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, indicate that the prolonged suppression produced by 7α-APTADD is not due to clearance of the compound from the cultures.

CONCLUSION

The introduction of bulky substitutions at the C-7 position of the B-ring of androstenedione has provided several very potent aromatase inhibitors. 7α-APTA is a very effective competitive inhibitor, with an apparent K_i of 18 nM. An effective mechanism-based inhibitor of aromatase, 7α-(4′-amino)phenylthio-1,4-androstadiene-3,17-dione (7α-APTADD) with an apparent K_i of 9.9 nM, was also developed and has the most rapid rate of inactivation reported to date. Recent results from inhibitory studies of various 7-substituted 4,6-androstadiene-3,17-dione derivatives suggest that only those derivatives that can project the 7-aryl substituent into the 7α pocket are effective inhibitors. Overall, the most effective B-ring modified aromatase inhibitors are those with 7α-aryl derivatives, with several analogs having 2–10 times greater affinity for the enzyme than the substrate. These structure–activity relationships suggest that additional interactions occur between the phenyl ring at the 7α-position and amino acids at or near the enzymatic site of aromatase to result in enhanced affinity of the inhibitors.

In addition, these 7α-substituted and 7-substituted C_{19} steroids inhibit aromatase activity present in both MCF-7 mammary carcinoma cells and in JAr choriocarcinoma cells. 7α-APTADD and 7α-APTA were the most effective inhibitors of these analogs; similar inhibition was observed with another potent steroidal agent, 4-OHA. In JAr cell cultures, 7α-APTADD and 4-OHA demonstrated the greatest efficacy. Furthermore, 7α-APTADD produced prolonged suppression of aromatase activity in the JAr cell culture system, suggesting enzyme-mediated inactivation of aromatase. Thus, these microsomal and cell culture studies of several potent 7α-substituted and 7-substituted C_{19} steroidal aromatase inhibitors encourage further development of this group of medicinal agents for the treatment of estrogen-dependent mammary carcinoma.

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