Biochemical Studies of Estr-4-ene-3,6,17-trione and 5 α -Androstan-17-ones with or without a Carbonyl Function at C-3 and/or C-6 as Aromatase Inhibitors

Mitsuteru NUMAZAWA,* Ayako MUTSUMI, Mii TACHIBANA, and Masao NAGAOKA

Tohoku College of Pharmacy, 4-1 Komatsushima 4-chome, Aobaku, Sendai 981, Japan.

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19-Nor (2) and 5 α -reduced (3) derivatives of androst-4-ene-3,6,17-trione (1) as well as 5 α -androstan-17-ones 4–6 were tested for their abilities to inhibit aromatase in human placental microsomes. All the steroids except 5 α -6-one 4 were fair to good competitive inhibitors of the enzyme, with apparent K_i 's ranging from 50 to 820 nM in which 5 α -3-one 5 was the most potent among them. The inhibitory activities of the 19-nor and 5 α -reduced derivatives (2 and 3) were less potent than that of the parent compound 1. Inhibitor 2 caused a time-dependent, pseudo-first-order inactivation of aromatase activity with a rate constant for inactivation of 0.148 min^{-1} in the presence of NADPH in air. The substrate androstenedione prevented the inactivation and L-cysteine did not protect aromatase from the inactivation.

Key words aromatase; suicide substrate; estr-4-ene-3,6,17-trione; competitive inhibitor; 5 α -androstan-3,17-dione; 5 α -androstan-17-one

Aromatase, a unique cytochrome P-450 enzyme complex, catalyzes the conversion of androstenedione and testosterone to estrone and estradiol, respectively.¹⁾ The aromatization process is thought to proceed via three sequential oxygenations of the C-19 angular methyl of the androgen.²⁾ Compounds affecting the aromatase inhibition have potential applications in the treatment of estrogen-dependent breast cancer.³⁾

Androst-4-ene-3,6,17-trione (1) is one of the earliest discovered suicide substrates.⁴⁾ We^{4c,5)} and Covey's group^{4b)} have studied the mechanism of aromatase inactivation by steroid 1. Two sequential hydroxylations initially occur at C-19 to give a 19-oxo steroid through the 19-hydroxy derivative. Further oxygenation yields a reactive electrophile that immediately binds to the active site in an irreversible manner, resulting in the inactivation of aromatase. In this sequence, 6-oxoestrogen is also produced by a mechanism similar to that involved in the aromatization of the natural substrate.^{5c)} Structure-activity relationships of A- or B-ring-substituted,⁶⁾ 19-substituted,⁷⁾ 3 α - and 3 β -reduced,⁸⁾ and 3-deoxy^{8a)} derivatives of inhibitor 1 have been extensively studied as aromatase inhibitors to show that an oxygen function at C-3 is essential to the time-dependent inactivation caused by a 4-en-6-one steroid. To gain further insight into the structural requirement necessary for aromatase inhibition by 3,6-dione steroids, this study was focused on aromatase inhibition by 19-nor- and 5 α -reduced derivatives (2 and 3) of inhibitor 1 as well as 3- and/or 6-deoxy derivatives (4–6) of the 5 α -steroid 3. These steroids inhibited human placental aromatase in a competitive manner. The nor steroid 2 caused a time-dependent inactivation of the enzyme.

MATERIALS AND METHODS

Materials Androst-4-ene-3,6,17-trione (1),^{4c)} 5 α -androstan-3,6,17-trione (3),⁹⁾ 5 α -androstan-6,17-dione (4),¹⁰⁾ and 5 α -androstan-17-one (6)¹¹⁾ were synthesized

according to the methods previously reported. 5 α -Androstan-3,17-dione (5) and estr-4-ene-3,17-dione (7, 19-norandrostenedione) were purchased from Sigma Chemical (St Louis, MO), [1 β -³H]androstenedione (24.1 $\mu\text{Ci}/\text{mmol}$) (³H distribution: $\beta/\alpha=69.8/30.2$) from New England Nuclear Corp. (Boston, MA), and NADPH from Kohjin Co., Ltd. (Tokyo, Japan).

Estr-4-ene-3,6-17-trione (2) A solution of potassium *tert*-butoxide (80 mg, 0.71 mmol) in *tert*-butanol (8 ml) was added to a solution of estr-4-ene-3,17-dione (7) (100 mg, 0.37 mmol).¹²⁾ The mixture was stirred at room temperature for 40 min in air, diluted with ethyl acetate (100 ml), washed with water, and dried over Na₂SO₄. Evaporation of the solvent gave an oil which was purified by preparative thin-layer chromatography (silica gel plate: E. Merck AG, Darmstadt, Germany; solvent: hexane–ethyl acetate (1:1)). Two major products were isolated by the chromatography. The more polar product ($R_f=0.5$) was recrystallized from acetone to afford compound 2 (13 mg, 13%): mp 132–134 °C. ¹H-NMR (270 MHz, CDCl₃) δ : 0.95 (3H, s, 18-Me), 6.52 (1H, dd, $J=2.9, 1.1$ Hz, 4-H). IR (KBr): 1744, 1694, 1674 (C=O) cm⁻¹. UV (95% EtOH) λ_{max} (ϵ): 253 nm (8200). Exact MS found 286.1560, calculated for C₁₈H₂₂O₃ 286.1590.

The less polar product ($R_f=0.6$) was recrystallized from methanol to give 6-oxoestrone (3.3 mg, 3%): mp 228–230 °C (lit.¹³⁾ 234–238 °C). ¹H-NMR (270 MHz, CDCl₃) δ : 0.93 (3H, s, 18-Me), 7.07 (1H, dd, $J=8.6, 2.9$ Hz, 2-H), 7.29 (1H, d, $J=8.6$ Hz, 1-H), 7.56 (1H, d, $J=2.9$ Hz, 4-H).

Enzyme Preparation Human placental microsomes (particles sedimenting at 105000 $\times g$ for 60 min) were obtained by the method reported by Ryan.¹⁴⁾ They were washed once with 0.05 M dithiothreitol solution, lyophilized, and stored at –20 °C. No significant loss of aromatase activity occurred for two months.

Aromatase Assay Procedure Aromatase activity in the placental microsomes was determined essentially according to the known radiometric method¹⁵⁾ in which tritium was released from [1 β -³H]androstenedione aromatization

* To whom correspondence should be addressed.

Chart 1

to form tritiated water in the incubation medium. The screening assay for determination of the IC₅₀ value, the kinetic assay, and the time-dependent assay were carried out essentially according to the assay methods described in our previous work.^{5a)} Briefly, 20 μ g protein of the lyophilized microsomes, 300 nM of the [³H]androstenedione, and a 20-min incubation time for the screening assay and 5-min incubation time for the kinetic assay, respectively, were employed in this study, and the assays were carried out in 67 mM phosphate buffer, pH 7.5, in the presence of NADPH (150 μ M) in air. In the time-dependent inactivation experiment, 0, 0.5, 1.0 and 2.0 μ M of inhibitor 2 were incubated with or without androstenedione (2 μ M), NADPH (150 μ M) and L-cysteine (0.5 mM) at 37°C with the placental microsomes (200 μ g protein) in the phosphate buffer in air, then 1/10 of the incubation mixture was used for assay of the remaining aromatase activity under the screening assay conditions.

RESULTS

Reversible inhibition of aromatase activity by the 19-nor steroid 2 and the 5 α -steroids 3–6 with or without a carbonyl function at C-3 and/or C-6, were tested *in vitro* by enzyme kinetics studies using human placental microsomes. The parent steroid 1 and 19-norandrostenedione (7) are listed for comparison. The results are shown in Table 1. IC₅₀'s were initially obtained under initial velocity conditions. Then, in order to characterize the nature of their binding to the active site of aromatase, aromatization was measured at several concentrations of the inhibitors and androstenedione. The results of the studies were plotted in a typical Lineweaver–Burk plot. All the steroids studied, except steroid 4, of which the kinetics of inhibition were not carried out because of its poor inhibitory activity (IC₅₀ value, > 50 μ M), exhibited clear-cut competitive-type inhibition. The apparent inhibition constants (*K*_i's), an index of the affinity of the enzyme for the inhibitor, were determined by analysis of the Dixon plot. The Lineweaver–Burk plot of aromatase inhibition by compound 2 is shown in Fig. 1. In these studies, the apparent *K*_m for androstenedione was found to be about 20 nM.

Inhibitors 2 and 3 were then tested for their ability to cause a time-dependent inactivation of aromatase. The 19-nor-4-ene-3,6-dione 2 showed a time-dependent loss of aromatase activity when incubated in the presence of NADPH, whereas the 5 α -3,6-dione 3 at the concentrations

Table 1. *In Vitro* Aromatase Inhibition Activity of 19-Nor and 5 α -Reduced Steroids

Inhibitor	IC ₅₀ , ^{a)} μ M	<i>K</i> _i , ^{b)} μ M	Inhibition ^{c)}
19-Nor-4-ene-3,6-dione 2	12	820	Competitive
5 α -3,6-Dione 3	4.0	300	Competitive
5 α -6-One 4	> 50	ND ^{d)}	ND
5 α -3-One 5	0.90	50	Competitive
5 α -17-One 6	2.3	200	Competitive
For comparison			
4-Ene-3,6-dione 1	0.28	24	Competitive
19-Nor-4-en-3-one 7	1.2	140	Competitive ^{e)}

a) [³H] Androstenedione, 0.3 μ M; human placental microsomes, 20 μ g protein; incubation time, 20 min. b) An apparent *K*_i value was obtained by a Dixon plot. c) Inhibition type was determined by a Lineweaver–Burk plot. d) ND; not determined. e) Reference 19.

Fig. 1. Lineweaver–Burk Plot of Inhibition of Human Placental Aromatase by 19-Nor-4-ene-3,6-dione 2 Using Androstenedione as a Substrate

Each point represents the mean of two determinations which varied by less than 10% of the mean. Inhibition experiments with all the other steroids examined gave essentially similar plots to Fig. 1 (data not shown).

employed (0.3, 0.6, and 1.0 μ M) did not. Pseudo-first-order kinetics were obtained during the first 12 min of the incubation of inhibitor 2 when the kinetic data were analyzed according to the method of Kitz and Wilson¹⁶⁾ (Fig. 2). A double-reciprocal plot of *k*_{obs} versus inhibitor concentration gave a *K*_i¹⁷⁾ value of 850 nM and a rate constant for inactivation (*k*_{inact} value) of 0.148 min^{−1},

Fig. 2. Time-Dependent Inactivation (A) and Concentration-Dependent Inactivation (B) by 19-Nor-4-ene-3,6-dione **2** in the Presence of NADPH in Air

Concentrations of the inhibitor: control (0 μM), $\bullet$; 0.5 μM , $\blacksquare$; 1.0 μM , $\blacktriangle$; 2.0 μM , $\times$. Each point represents the mean of two determinations which varied by less than 10% of the mean.

respectively.

NADPH was essential for the time-dependent inactivation by the irreversible inhibitor (Fig. 3A). The substrate androstenedione blocked the inactivation while a nucleophile, L-cysteine, had no significant effect on it (Fig. 3B and 3C).

DISCUSSION

We carried out a series of experiments using the 19-nor-4-ene-3,6-dione **2** and the 5 α -3,6-dione **3**, as well as the deoxy derivatives (**4**–**6**) of the latter, to determine the structural features required for aromatase inhibition. The 5 α -3-one **5** was a good competitive inhibitor ($K_i = 50$ nM), whereas the 19-nor steroid **2** and the 5 α -steroids (**3** and **6**) were fair (K_i : 820 nM for **2**, 300 nM for **3**, and 200 μM for **6**). On the other hand, the 5 α -6-one **4**, a regioisomer of compound **5**, was a poor inhibitor ($\text{IC}_{50} > 50$ μM). Schwarzel *et al.* have previously reported, without kinetic data, the inhibition of aromatase by steroid **5**.^{4a)} However, this is the first time that steroid **5** has been proved to be a good competitive inhibitor of the enzyme based on the kinetic assay.

The inhibitory activities of the 5 α -steroids **3** and **5** are lower, to a great extent, than those of the corresponding 4-ene steroids, **1** and androstenedione [K_i : 300 vs. 24 nM for **3** vs. **1**, 50 vs. 20 nM (K_m) for **5** vs. androstenedione], indicating that a double bond at C-4,5 of steroid **1** and androstenedione is essential for a tight binding to the active site of aromatase. Comparing the inhibitory activity of the 5 α -3-one **5** with that of the 6-one isomer **4**, it is suggested that the C-3 carbonyl, rather than the C-6-carbonyl, of the 5 α -3,6-dione **3** plays an important role in the binding of inhibitor **3** to the active site. It should be noted that

Fig. 3. Inactivation of Human Placental Aromatase by 19-Nor-4-ene-3,6-dione **2** under Various Conditions

(A) in the absence of NADPH, the inhibitor at concentrations of 1.0 μM ($\times$), and 2.0 μM ($\blacktriangle$) failed to produce the inactivation. Control sample contained no inhibitor ($\bullet$). (B) Androstenedione at a concentration of 2 μM ($\blacksquare$) protected aromatase from inactivation caused by the inhibitor (1 μM) without androstenedione ($\blacktriangle$) in the presence of NADPH. Control sample with ($\bullet$) or without ($\times$) androstenedione contained no inhibitor. (C) In the presence ($\blacksquare$) or absence ($\blacktriangle$) of L-cysteine (0.5 mM), a pseudo-first-order inactivation of aromatase by the inhibitor (1 μM) was observed. Control sample with ($\times$) or without ($\bullet$) L-cysteine contained no inhibitor. Each point represents the mean of two determinations which varied less than 10% of the mean.

5 α -androst-17-one (**6**), having no carbonyl group in the A/B ring system, was a fair competitive inhibitor of the enzyme ($K_i = 200$ nM). Similar structure-activity relationships have also been obtained in a series of androst-17-ones (K_i : 37 nM for androst-4-en-17-one¹⁸⁾ and 120 nM for androst-5-en-17-one¹⁹⁾). The 17-oxo steroids (**6** and its 4-ene and 5-ene derivatives) may be useful as conformational and catalytic probes for the active site of aromatase, though their binding geometries to the active site is not clear. It has previously been reported that compound **6** does not inhibit androstenedione aromatization.^{4a)} The previous result is not reliable because the inhibition experiment was not carried out under the initial velocity

conditions which were employed in this study. The 19-nor-4-ene-3,6-dione **2** was a less potent inhibitor than the corresponding 19-methyl steroid **1** ($K_i = 24$ nM) in analogy with a series of androstenediones (see Table 1). A 19-methyl group of a 4-en-3-one steroid would be essential for its tight binding to the enzyme.

The 19-nor steroid **2** inactivated the aromatization of androstenedione in a time-dependent, pseudo-first-order manner only in the presence of NADPH in air; however, its 5 α -derivative **3** did not. A double-reciprocal plot of the apparent rate constant for inactivation *versus* the concentration of the steroid **2** was linear. This indicates the formation of a dissociable enzyme-inhibitor complex followed by unimolecular inactivation. The apparent K_i (850 nM) obtained by the Kitz-Wilson plot is similar to the apparent K_i (820 nM) obtained from the competitive experiment, suggesting the initial binding of the inhibitor to the enzyme is not rate-limiting in the inactivation process.

The rate of inactivation decreased when the substrate androstenedione was included in the incubation mixture. In the nucleophile protection experiment, L-cysteine failed to protect aromatase from inactivation by the inhibitor. Thus, covalent-bond formation between the enzyme and the reactive intermediate appears to occur rapidly at the active site, therefore preventing diffusion of the activated inhibitor, a reactive electrophile, into the surrounding media.

It has recently been proposed that 19-nor steroid having a 5-en-7-one structure inactivates aromatase in a suicide manner, probably through 1 β -hydroxylation, whereas the 19-nor steroid **7**, having a 4-en-3-one structure, does not.^{19,20} Considering the previous findings, although there is no direct evidence, it is presumed that the 1 β -hydroxylation may be involved in the aromatase inactivation by the 19-nor-4-ene-3,6-dione **2**, similarly to that by the 19-nor-5-en-7-one steroid. The 1 β -hydroxy derivative, first produced by catalysis of aromatase, is converted into 1(10),4-diene-3,6-dione through further oxygenation by the enzyme or nonenzymatic dehydration. The diene intermediate covalently binds to the enzyme in a 1,6-addition manner to result in the irreversible inactivation of the enzyme. Further study on the inactivation mechanism is currently underway in our laboratory.

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