Kinetic Analysis of Reversible Inhibition of 16 α -Hydroxyandrostenedione Aromatization in Human Placental Microsomes by Suicide Substrates of Androstenedione Aromatization

Mitsuteru NUMAZAWA,* Ayako MUTSUMI, Mii TACHIBANA, and Akiko YOSHIMURA

Tohoku Pharmaceutical University; 4-4-1 Komatsushima, Aoba-ku, Sendai 981-8558, Japan.

Received December 4, 2002; accepted March 6, 2003

To gain insight into the catalytic function of aromatase and its substrate specificity, we studied reversible inhibition of 16 α -hydroxyandrostenedione (16 α -OHAD) aromatization in human placental microsomes by several suicide substrates of androstenedione (AD) aromatization, including 4-hydroxyAD (1), 6-oxoAD (2) and its 19-hydroxy analogue 3, androst-5-ene-4,7,17-trione (4), and 10 β -acetoxyandrost-5-en-7,17-dione (5) that, in contrast, do not cause a suicide inactivation of 16 α -OHAD aromatization. All inhibitors examined blocked 16 α -OHAD aromatization in a competitive manner with apparent K_i values ranging from 0.50 to 980 nM. The relative K_i values between inhibitors 1–5 obtained in the 16 α -OHAD aromatization experiments were markedly different from those obtained in the AD aromatization experiments. The results predict that all inhibitors examined bind to the 16 α -OHAD binding site in a manner that does not cause suicide inactivation of 16 α -OHAD aromatization. These findings would be useful for understanding the active (binding) site structure as well as the catalytic function of aromatase.

Key words human placental aromatase; suicide substrate; binding site; 16 α -hydroxyandrostenedione aromatization; reversible inhibition

Aromatase is a cytochrome P-450 enzyme responsible for the conversion of androstenedione (AD) and 16 α -hydroxyandrostenedione (16 α -OHAD) into estrogens, estrone, and 16 α -hydroxyestrone, respectively.^{1–3)} 16 α -OHAD rather than AD is a principal precursor of estrogen production in late pregnancy in the human placenta.⁴⁾ Inhibitors of aromatase are valuable as therapeutic agents in the treatment of estrogen-dependent breast cancer.⁵⁾ The human term placenta provides a plentiful source of aromatase for testing inhibitors.

4-Hydroxy AD (1)⁶⁾ and 6-oxo AD (2)⁷⁾ are one of the earliest discovered suicide substrates of AD aromatization in human placental microsomes. Moreover, the 19-hydroxy derivative of inhibitor 2, compound 3,⁸⁾ androst-5-ene-4,7,17-trione (4),⁹⁾ and 10 β -acetoxyestr-5-ene-7,17-dione (5),¹⁰⁾ also inactivate AD aromatization in placental microsomes in a suicide manner (Fig. 1). We recently reported that none of these five suicide substrates cause a time-dependent inactivation of 16 α -OHAD aromatization in placental microsomes in the presence of NADPH in air.¹¹⁾ In addition, it is known that 16 α -OHAD is a noncompetitive inhibitor of AD aromatization in placental microsomes.^{12–14)} It was also reported that AD inhibits aromatization of 16 α -OHAD in a noncompetitive manner.¹⁴⁾ AD, but not 16 α -OHAD, protected against the 4-hydroxy steroid 1-mediated suicide inactivation of 16 α -OHAD aromatization.¹⁴⁾

Taken together, it was interesting to determine whether inhibitors 1–5 inhibit 16 α -OHAD aromatization in a competitive manner or in a noncompetitive manner to understand the nature of the active (binding) site of aromatase. All steroids 1–5 blocked 16 α -OHAD aromatization in placental microsomes in a competitive manner.

MATERIALS AND METHODS

[1 β -³H]AD (27.5 Ci/mmol; ³H-distribution, 1 β =74–79%)

was purchased from New England Nuclear Corp. (Boston, MA, U.S.A.), and NADPH from Kohjin Co., Ltd. (Tokyo, Japan). [1 β -³H]16 α -OHAD (715 mCi/mmol) was microbiologically prepared from commercially available [1 β -³H]AD with *Streptomyces roseochromogenes* as previously described by us.¹⁵⁾ 4-Hydroxy AD (1), 6-oxo AD (2), 19-hydroxy-6-oxo AD (3), androst-5-ene-4,7,17-trione (4), and 10 β -acetoxyandrost-5-ene-7,17-dione (5) were synthesized according to the previously reported methods.^{6–10)}

Preparation of Placental Microsomes Human term placental microsomes (particles sedimenting at 10500 $\times g$ for 60 min) were obtained as described by Ryan.¹⁶⁾ They were washed once with 0.5 mM dithiothreitol solution, lyophilized, and stored at –20 °C. No significant loss of aromatase activity occurred over the period of the study (2 months).

Aromatase Assay Procedure Aromatase activity was measured according to the procedure of Siiteri and Thompson,¹⁷⁾ in which tritiated water released from [1 β -³H]AD or [1 β -³H]16 α -OHAD into the incubation medium during aromatization was used as an index of enzyme activity. All enzymatic studies were carried out in 67 mM phosphate buffer, pH 7.5, at a final incubation volume of 0.5 ml under initial velocity conditions.^{9,15)} The incubation mixture for the IC₅₀ experiment contained 180 μ M of NADPH, 300 nM of [1 β -³H]AD, or 400 nM of [1 β -³H]16 α -OHAD, 20 μ g protein (for the [1 β -³H]AD aromatization experiment) or 40 μ g protein (for the [1 β -³H]16 α -OHAD aromatization experiment) of the lyophilized microsomes, various concentrations of steroids 1–5, and 25 μ l of MeOH, and the entire mixture was incubated at 37 °C for 20 min in the experiment with [1 β -³H]AD or for 30 min in the experiment with [1 β -³H]16 α -OHAD. For the kinetic assay experiment, the incubation mixture contained 180 μ M of NADPH, 16, 28, or 34 nM of [1 β -³H]AD or 48, 73, or 105 nM of [1 β -³H]16 α -OHAD, 20 μ g protein (for the AD aromatization) or 40 μ g protein (for the 16 α -OHAD aromatization) of the microsomes, vari-

* To whom correspondence should be addressed. e-mail: numazawa@tohoku-pharm.ac.jp

ous concentrations of steroids **1**–**5**, and 25 μ l of MeOH. The mixture was incubated at 37 °C for 5 min in the [1β - ^{3} H]AD experiment or for 30 min in the [1β - ^{3} H]16 α -OHAD experiment. The rate of 16 α -OHAD aromatization was about one-third that of AD aromatization, and thus an increased amount of microsomal protein (40 μ g) and prolonged incubation time (30 min) were employed in the experiments with [1β - ^{3} H]16 α -OHAD. Apparent K_i values were calculated using nonlinear regression analysis with GraFit software.¹⁸⁾

RESULTS AND DISCUSSION

4-Hydroxy AD (**1**), 6-oxo AD (**2**) and its 19-hydroxy derivative **3**, 5-ene-4,7-dione **4**, and 10 β -acetoxy-5-en-7-one **5**, which were previously evaluated as suicide substrates of AD aromatization in human placental microsomes, were tested as reversible inhibitors of 16 α -OHAD aromatization in this study (Fig. 1). Table 1 summarizes IC_{50} and apparent K_i values for inhibitors **1**–**5** obtained by a radiometric assay using [1β - ^{3} H]16 α -OHAD as a substrate. For comparison, the values were also obtained for AD aromatization and listed in Table 1. In the assays, apparent K_m and V_{max} values for AD or 16 α -OHAD were 33 nM and 120 pmol/min/mg protein or 580 nM and 36 pmol/min/mg protein, respectively. IC_{50} values were first obtained and then, to characterize the nature of their binding to the active site of aromatase, aromatization was measured in the presence of several concentrations of inhibitors and the substrate 16 α -OHAD or AD. The results of the studies were plotted on typical Lineweaver–Burk plots. All of the inhibitors examined exhibited clear-cut competitive-type inhibition of 16 α -OHAD aromatization. The results of the assay for powerful to good inhibitors (**1**–**4**) are shown in Fig. 2. The apparent inhibition constant (K_i), an index of the affinity of the enzyme for the inhibitor, was determined by analysis of a Dixon plot in each case.

The K_i values obtained in the 16 α -OHAD aromatization experiments were fairly low compared with those obtained in the corresponding AD aromatization experiments. This should be due to the fact that the 16 α -hydroxy substrate [1β - ^{3} H]16 α -OHAD binds to the active site of aromatase with a lower affinity than the other 16-deoxy substrate, [1β - ^{3} H]AD, (K_m : 580 nM for 16 α -OHAD vs. 33 nM for AD). The relative K_i values between inhibitors **1**–**5** (1 : 12 : 140 : 60 : 1960 for **1** : **2** : **3** : **4** : **5**) in the series of 16 α -OHAD aromatization experiments were markedly different from those (1 : 1.3 : 44.4 : 20 : 666 for **1** : **2** : **3** : **4** : **5**) in another series of experiments with AD as a substrate. It is noteworthy that the 4-hydroxy steroid **1** is an extremely powerful inhibitor of 16 α -OHAD aromatization. It has previously been reported that in the inhibition study of placental aromatase with 3-deoxy- and 6-oxo-steroids, the relative inhibitory activities obtained using 16 α -OHAD as a substrate are different from those obtained using AD as a substrate.¹⁹⁾

It is now believed that a single enzyme species catalyzes the aromatization independent of the difference in the D-ring substitution of androgens (AD vs. 16 α -OHAD).^{1–3,19)} The previous findings^{12–14,20)} have supported the idea of two binding sites for one enzyme in placental microsomes, *i.e.*, one site for AD aromatization and a second for 16 α -OHAD aromatization. Inhibitors **1**–**5**, which inactivate AD but not 16 α -OHAD aromatization in a suicide manner, blocked not

Fig. 1. Structures of Aromatase Inhibitors

Table 1. Reversible Inhibition of 16 α -OHAD Aromatization (Method A) and AD Aromatization (Method B) by Inhibitors

Inhibitor	$IC_{50}^a)$ (nM)		$K_i^b)$ (nM)	
	Method A	Method B	Method A	Method B
4-OHAD (1)	0.72	180	0.50	18
6-Oxo AD (2)	15	280	6.0	24
19-Hydroxy steroid 3	165	4200	70	800
4,7-Dione steroid 4	60	3600 ^{c)}	30	430 ^{c)}
10 β -Acetoxy steroid 5	2800	92000 ^{d)}	980	12000 ^{d)}

a) 400 nM of [1β - ^{3} H]16 α -OHAD, 40 μ g of placental microsomal protein, and 30-min incubation time were used in Method A. 300 nM of [1β - ^{3} H]AD, 20 μ g of microsomal protein, and 20-min incubation time were used in Method B. b) Apparent inhibition constant (K_i) was obtained by Dixon plot. All inhibitors examined showed competitive inhibition of 16 α -OHAD aromatization (Method A) and AD aromatization (Method B) based on analysis of the Lineweaver–Burk plot. c) Ref. 9. d) Ref. 10.

only AD aromatization but also 16 α -OHAD aromatization in a competitive manner. Taken together, the results indicate that the suicide inactivators of AD aromatization would bind to both binding sites but the inactivation reaction, the aromatase-catalyzed production of a reactive electrophile that binds to a nucleophilic residue in the active site of the enzyme, would not occur at the 16 α -OHAD site. It is known that 6-oxoAD (**2**) inactivates AD aromatization in a suicide manner through two subsequent 19-hydroxylations followed by an epoxidation at the C-4 and C-5 positions, producing a 19-oxo-4 β ,5 β -epoxy intermediate as the reactive electrophile.^{21,22)} In addition, it is speculated that the 19-oxygenated intermediate may play a role as the reactive electrophile in the inactivation of AD aromatization by 4-hydroxyAD (**1**).²³⁾ Based on those previous findings, it is likely that binding manners of inhibitors **1** and **2** in the 16 α -OHAD site would not be appropriate for the aromatase-catalyzed 19-oxygenation in each case. Although the mechanisms of the inactivation of AD aromatization by the inhibitors **3**–**5** are currently unknown, they would also bind to the 16 α -OHAD site in a manner not suitable for the inactivation reaction. These findings will be valuable for understanding not only the nature of binding sites of aromatase, especially the

Fig. 2. Lineweaver-Burk Plots of the Inhibition of Human Placental Aromatase by 4-Hydroxy Steroid **1** (A), 6-Oxo Steroid **2** (B), 19-Hydroxy-6-oxo Steroid **3** (C), and 5-Ene-4,7-dione Steroid **4** (D) with [1β - ^{3} H] 16α -OHAD as a Substrate

Each point represents the mean of two determinations that varied by less than 5% of the mean. The inhibition experiment with inhibitor **5** gave an essentially similar result to those for **1**–**4** (data not shown).

nature of the 16α -OHAD site, but also the binding aspects of inhibitors (substrates) in the binding sites.

Acknowledgments This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sport, and Culture of Japan. We are grateful to Dr. Hideo Imaizumi of the Imaizumi Hospital, Sendai, for generously supplying human term placentas.

REFERENCES

- 1) Thompson E. A., Jr., Siiteri P. K., *J. Biol. Chem.*, **249**, 5373–5378 (1974).
- 2) Kellis J. T., Jr., Vickery L. E., *J. Biol. Chem.*, **262**, 4413–4420 (1987).
- 3) Yoshida N., Osawa Y., *Biochemistry*, **30**, 3003–3010 (1991).
- 4) Ryan K. J., "Maternal-Fetal Endocrinology," ed. by Tulchinsky D., Ryan K. J., W. S. Saunders Company, Philadelphia, 1980, pp. 3–6.
- 5) For example, see Johnson J. O., *Crit. Rev. Biochem. Mol. Biol.*, **33**, 375–405 (1998).
- 6) Brodie A. M. H., Garrett W. M., Hendrickson J. R., Tsai-Morris C. H., Marcotte P. A., Robinson C. H., *Steroids*, **38**, 693–702 (1981).
- 7) Covey D. F., Hood W. F., *Endocrinology*, **108**, 1597–1599 (1981).
- 8) Numazawa M., Mutsumi A., Hoshi K., Kigawa M., Oshibe M., *J. Steroid Biochem. Mol. Biol.*, **39**, 959–966 (1991).
- 9) Numazawa M., Tachibana M., Tateda Y., *J. Steroid Biochem. Mol. Biol.*, **58**, 431–438 (1996).
- 10) Numazawa M., Tachibana M., *Steroids*, **62**, 516–522 (1997).
- 11) Numazawa M., Tachibana M., Mutsumi A., Yoshimura A., Osawa Y., *J. Steroid Biochem. Mol. Biol.*, **81**, 165–172 (2002).
- 12) Canick J. A., Ryan K. J., *Mol. Cell. Endocrinol.*, **6**, 105–115 (1976).
- 13) Cantineau R., Kremers P., De Graeve J., Gielen J. E., Lambotte R., *J. Steroid Biochem.*, **16**, 157–163 (1982).
- 14) Purohit A., Oakey R. E., *J. Steroid Biochem. Mol. Biol.*, **33**, 439–448 (1989).
- 15) Numazawa M., Mutsumi A., Nakakoshi M., Nagaoka M., *Chem. Pharm. Bull.*, **40**, 1839–1842 (1992).
- 16) Ryan K. J., *J. Biol. Chem.*, **234**, 268–272 (1959).
- 17) Siiteri P. K., Thompson E. A., Jr., *J. Steroid Biochem.*, **6**, 317–322 (1975).
- 18) GraFit version 5.0.3, Erithacus Software Limited, Surrey, UK, 2001.
- 19) Simpson E., Lauber M., Demeter M., Stirling D., Rodgers R., Means G., Mahendroo M., Kilgore M., Mendelson C., Waterman M., *J. Steroid Biochem. Mol. Biol.*, **40**, 45–52 (1991).
- 20) Numazawa M., Mutsumi A., *J. Steroids Biochem. Mol. Biol.*, **44**, 199–201 (1993).
- 21) Numazawa M., Midzuhashi K., Nagaoka M., *Biochem. Pharmacol.*, **47**, 717–726 (1994).
- 22) Numazawa M., Mutsumi A., Tachibana M., *Biochem. Pharmacol.*, **52**, 1253–1259 (1996).
- 23) Furth P. S., Rosenberger J., Marcotte P. A., Robinson C. H., *J. Enzyme Inhibition*, **4**, 131–135 (1990).