[CANCER RESEARCH (SUPPL.) 42, 3312s-3314s, August 1982]
0008-5472/82/0042-0000$02.00

Overview of Recent Development of Aromatase Inhibitors[1]

Angela M. H. Brodie

Department of Pharmacology and Experimental Therapeutics, University of Maryland School of Medicine, Baltimore, Maryland 21201

Abstract

Since the first publication in 1973 concerning aromatase inhibitors, several effective compounds have been reported by a number of investigators. Our studies with 4-hydroxyandrostene-3,17-dione, 4-acetoxyandrostene-3,17-dione, and 1,-4,6-androstatrienedione indicate that these compounds cause rapid competitive inhibition of the enzyme. Aminoglutethimide binds competitively to cytochrome P-450 and inhibits a number of steroid hydroxylations but is more active as an aromatase inhibitor. 16α-Bromoandrogens and 7α-(4'-amino)phenylthioandrostenedione are also reported to be aromatase inhibitors. As yet, only some of these compounds have been evaluated *in vivo*, but all appear to be similarly effective in inhibiting aromatization in breast tumors *in vitro*. Recent interest has focused on enzyme inactivators or "suicide" inhibitors. Such compounds act as substrates for the enzyme but are converted by the normal catalytic mechanism of the enzyme to reactive intermediates. These then bind covalently to the active site of the enzyme causing loss of activity. Active site-directed inhibitors are usually quite specific and have long-lasting effects *in vivo*. A number of new compounds, as well as some of the above compounds, appear to be inactivators of aromatase and are potentially interesting as agents for hormone-dependent breast cancer therapy.

The initial publication by our group (32) in 1973 on aromatase inhibitors describes evaluation of a number of compounds, of which the most active series was C_{19} 3-ketosteroids. These compounds resemble the natural substrate of aromatase, androstenedione. The best inhibitors were found to be androstene-3,6,17-trione and ATD.[2]

As aromatization is a unique reaction and the last in the series of steps in the biosynthesis of steroids, it seems likely that compounds interacting with the aromatizing enzyme would be specific inhibitors. We envisaged that effective inhibitors of aromatase might have useful application for controlling estrogen-dependent processes in reproduction and in hormone-dependent breast cancer. In addition, such compounds may be useful tools for aiding studies on the mechanisms involved in estrogen biosynthesis and the role of estrogens in physiological processes. Commercially available ATD is now being used quite extensively for the latter purpose by a number of investigators.

Subsequently, we found that 4-OHA (15) and 4-acetoxyA (11) were also highly effective aromatase inhibitors. These and the above compounds all showed kinetics of rapid competitive inhibition of placental aromatase (15, 32).

As we planned to study *in vivo* activity of aromatase inhibitors in the rat, we developed a rat ovarian microsomal preparation with a high level of aromatase activity induced by treatment of the rats with pregnant mares' serum gonadotropin (14). Comparative studies with placental and ovarian microsomes indicated that both preparations of aromatase had similar properties. Most compounds inhibited both ovarian and placental aromatase to about the same extent. An exception was androstene-3,6,17-trione which was more active in the placental than in the ovarian system, suggesting that some differences may exist between the 2 enzymes.

Other investigators have evaluated compounds as aromatase inhibitors. The most active series of compounds studied by Siiteri and Thompson (34), Thompson *et al.* (36), and Thompson and Siiteri (37, 38) were 5α-reduced C_{19} steroids. Based on their observations, these investigators suggested that several androgen derivatives which had been used for many years in breast cancer treatment may be acting as aromatase inhibitors (36). One such compound is Δ^1-testololactone, a C_{19} steroid without androgenic activity. Subsequently, Barone *et al.* (4) demonstrated that testololactone-inhibited peripheral aromatization of androstenedione to estrone but not to estradiol in postmenopausal women with breast cancer. Extraovarian aromatization of androgens, mainly of adrenal origin, increases after menopause. Aromatization also occurs in some breast tumors (2, 26, 42). These peripheral sources of estrogen could contribute to tumor stimulation in postmenopausal breast cancer patients.

Another compound studied by Thompson and Siiteri (37, 38) was AG. This is a potent inhibitor of aromatase (37, 38) as well as an inhibitor of a number of other steroid hydroxylations. AG binds competitively to cytochrome P-450 at a site somewhat distant from the binding site for steroid substrates undergoing hydroxylation (type II binding rather than type I binding) (22, 40, 41). Inhibition of aromatase by AG appears to be greater than that by other hydroxylating enzymes. We have compared the activity of this compound and Δ^1-testololactone with 4-OHA for aromatase inhibition in placental and ovarian microsomes (Table 1). Testololactone is less effective than either AG or 4-OHA.

To date, AG (28, 29, 30, 31) and Δ^1-testololactone (21, 33) are the only aromatase inhibitors that have been studied in human subjects. AG, as well as Δ^1-testololactone, causes objective tumor regression in postmenopausal breast cancer patients. AG has also been demonstrated to inhibit peripheral conversion of androgens to estrogens (31).

However, extensive studies in animal models have been carried out with 4-OHA, 4-acetoxyA, and ATD (7, 11, 15). These latter compounds inhibit ovarian estrogen secretion and cause marked regression of hormone-dependent mammary tumors induced in the rat with carcinogens 7,12-dimethylbenz(a)anthracene (6, 12, 29) or *N*-nitrosomethylurea (3). In primates, 4-OHA and 4-acetoxyA also inhibit more than 90% of peripheral aromatization. For these studies, male rhesus

[1] Presented at the Conference "Aromatase: New Perspectives for Breast Cancer," December 6 to 9, 1981, Key Biscayne, Fla. This work was supported by Grants CA-18595 and HD-13909 from NIH.

[2] The abbreviations used are: ATD, 1,4,6-androstatriene-3,17-dione; 4-OHA, 4-hydroxyandrostene-3,17-dione; 4-acetoxyA, 4-acetoxyandrostenedione; AG, 3-(*p*-aminophenyl)-3-ethyl-2,6-piperidinedione.

Table 1

Compounds tested for competitive inhibition of aromatase

Microsomes were prepared from ovaries of rats primed for 12 days with pregnant mares' serum gonadotropin given as 100 IU/day s.c. on alternate days (14). Each incubation contained 50 mg wet weight (0.75 mg protein) of ovarian tissue, [1,2-^{3}H]androstenedione, test compound, and an NADPH-generating system in 2.5 ml phosphate buffer (pH 7.4) for 30 min at 37° and oxygen. All are mean values.

Compound	I:S[a]	% of inhibition of aromatization
Control		0
4-OHA	1	73
ATD	1	72
AG	3	60
	6	68
Teslac[b]	10	25
	20	34
	50	43
Control		0
4-OHA	1	71
	3	88
6-BrA	3	62
	6	74

[a] I:S, ratio of inhibitor concentration to substrate (0.28 and 0.7 μM androstenedione for first and second sets of data, respectively).

[b] Teslac, 17α-oxo-D-homo-1,4-androstadiene-3,17-dione; 6-BrA, 6-bromoandrostene-3,17-dione.

monkeys were given a constant infusion of radiolabeled androstenedione. After steady-state conditions were established, blood samples were drawn and the conversion to estrone and estradiol was measured in the resulting plasma. No alteration in the metabolic clearance rates of androstenedione and estrone or in the interconversion of androstenedione to testosterone, of androstenedione to dihydrotestosterone, and of estrone to estradiol was observed in this experiment (10). As with testololactone, these inhibitors appear to specifically affect aromatization.

A series of 7α-substituted C_{19} steroids have been evaluated as competitive inhibitors by Brueggemeier *et al.* (17). The most effective inhibitor of this series was 7α-(4'-amino)phenylthioandrostenedione.

As mentioned above, studies by a number of investigators have demonstrated that a significant portion of human breast tumors aromatize androstenedione to estrogens. Abul-Hajj *et al.* (1) recently compared *in vitro* inhibition of aromatization in human breast tumors by 7α-(4'-amino)phenylthioandrostenedione, aminoglutethimide, and 4-OHA and found them all similarly effective in causing 81 to 97% inhibition, although at rather high concentrations of inhibitors.

Recently, several groups have been interested in developing enzyme inactivators or "suicide" inhibitors of aromatase. Such compounds act as substrates for the enzyme but are converted by the normal catalytic mechanisms of the enzyme to reactive intermediates. These then bind covalently to the active site of the enzyme, causing loss of activity. Suicide inhibitors are normally quite specific and have long-lasting effects *in vivo*. Inhibitors of this type have been successfully used for other enzymes (35).

A number of active site-directed inhibitors were reported by Bellino *et al.* (5). These compounds were bromoandrogens and were prepared for affinity labeling aromatase to aid in studies on estrogen biosynthesis. Bellino *et al.* (5) demonstrated that the most active compound 6α-bromoandrostenedione acts both as a competitive inhibitor and as an inactivator of aromatase, indicating that the compound goes to the same enzyme site as androstenedione. The activity of this compound is compared in Table 1.

More recent studies of suicide substrates involve compounds modified at the C-19 position. To date, 10β-propargyl-4-estrene-3,17-dione is the most active compound reported in this series (19, 23, 25). Interestingly, 4-OHA, 4-acetoxyA, ATD, and androstene-3,6,17-trione (7, 9, 18) also inactivate aromatase but rather more slowly than they compete for the enzyme. Kinetic studies show that loss of enzyme activity is a time-dependent first-order process which occurs more slowly in the placenta than ovary. 4-OHA and 10β-propargyl compound have equivalent activities as inactivators of ovarian aromatase ($t_{1/2} \sim 20$ min). Enzyme inactivation occurs more slowly in both tissues than competitive inhibition. The 2 compounds also appear to be equally effective inhibitors of aromatase in breast tumor incubations (27).

In our inactivation studies with 4-OHA, aromatase activity was not regained after microsomes preincubated with 4-OHA and then treated with charcoal and exhaustive washing (which were considered sufficient to remove any residual 4-OHA) had been allowed to stand 18 hr at 0°. The addition of androstenedione to the preincubation with 4-OHA protected the aromatase from inactivation, suggesting involvement of the active site of the enzyme in the inhibitory process. When control (untreated) and 4-OHA-preincubated microsomes were mixed in equal proportions and the aromatase activity was assayed, the activity was similar to the control value. The same result was obtained when boiled microsomes were mixed with control microsomes. These findings suggest that 4-OHA causes long-term inactivation or irreversible inhibition of aromatase.

Aromatase inactivation may explain the sustained effects of 4-OHA observed *in vivo* and the high efficacy of the compound in view of our findings that 4-OHA is cleared rapidly from the circulation. Thus, in the monkey, the disappearance of 4-OHA from the blood following an i.v. injection of [6,7-^{3}H]-4-OHA was very rapid, even faster than androstenedione (13).

If the binding of these inhibitors is indeed irreversible, as it appears to be, the aromatization of the compound itself to an estrogen would be unlikely. We have investigated the metabolism of 4-OHA in the pregnant mares' serum-stimulated rat ovary and found that there was minimal conversion (less than 0.1%) of [6,7-^{3}H]-4-OHA to 4-hydroxyestrone (24). No other estrogens could be detected during the incubation. Inhibitors binding at the active site of the enzyme would also be expected to be quite specific. In studies with 4-OHA, no effect on other enzyme systems involved in the metabolism of progesterone or androstenedione was observed. Only at concentrations of 70 μM, 100 times that needed to inhibit aromatase, were 17β-hydroxysteroid dehydrogenase and 5α-reductase inhibited by 4-OHA, 4-acetoxyA, or ATD (8, 27). Similarly, as indicated above, these enzymes were unaffected by inhibitor treatment *in vivo* in studies with rhesus monkeys (10).

We have examined a number of other aromatase inhibitors we have prepared as enzyme inactivators. Thus, although 4-OHA and 4-acetoxyA cause inactivation, 4-methoxyandrostenedione and 4-chloroandrostenedione are without activity but act as competitive inhibitors. On the other hand, 4-hydroxy-1,4-androstenedione-3,17-dione and 4-hydroxy-4,6-androstenedione-3,17-dione inactivate ovarian aromatase but rather more slowly than 4-OHA.

Although the mechanism of the aromatase inactivation is as

yet unknown, studies into the structure-activity relationship of these varied compounds may aid in elucidating the mechanisms involved in aromatization as well as producing valuable new drugs for breast cancer treatment.

References

1. Abul-Hajj, Y. J. Inhibition of androgen aromatization in human breast cancer. J. Steroid Biochem., *13:* 1395–1400, 1980.
2. Abul-Hajj, Y. J., Iverson, R., and Kiang, D. T. Aromatization of androgens by human breast cancer. Steroids, *33:* 205–221, 1979.
3. Arafah, B., Gullino, P. M., Manni, A., and Pearson, O. H. Effect of ovariectomy on hormone receptors and growth of *N*-nitrosomethylurea-induced mammary tumors. Cancer Res., *40:* 4628–4630, 1971.
4. Barone, R. M., Shamonki, I. M., Siiteri, P. K., and Judd, H. L. Inhibition of peripheral aromatization of androstenedione to estrone in postmenopausal women with breast cancer using Δ^1-testololactone. J. Clin. Endocrinol. Metab., *49:* 672–676, 1979.
5. Bellino, F. L., Gilani, S. S. H., Eng, S. S., Osawa, Y., and Duax, W. L. Active-site inactivation of aromatase from human placental microsomes by brominated androgen derivatives. Biochemistry, *15:* 4730–4736, 1976.
6. Brodie, A. M. H. Inhibition of estrogen biosynthesis: an approach to treatment of estrogen-dependent cancer. Horm. Cancer Res., *15:* 507–514, 1981.
7. Brodie, A. M. H., Brodie, H. J., Garrett, W. M., Hendrickson, J. R., Marsh, D. H., and Tsai-Morris, C. H. The effect of aromatase inhibitor: 1,4,6-androstenedione (ATD) on DMBA-induced mammary tumors in the rat and its mechanism of action *in vivo*. Biochem. Pharmacol., in press, 1982.
8. Brodie, A. M. H., Brodie, H. J., Romanoff, L., Williams, J. G., Williams, K. I. H., and Wu, J. T. Inhibition of estrogen biosynthesis and regression of mammary tumors by aromatase inhibitors. Adv. Exp. Med. Biol., *138:* 179–190, 1982.
9. Brodie, A. M. H., Garrett, W. M., Hendrickson, J. R., Marcotte, P. A., and Robinson, C. H. Inactivation of aromatase activity in placental and ovarian microsomes by 4-hydroxyandrostenedione and 4-acetoxy-androstenedione. Steroids, *38:* 693–702, 1981.
10. Brodie, A. M. H., and Longcope, C. Inhibition of peripheral aromatization by aromatase inhibitors, 4-hydroxy- and 4-acetoxyandrostenedione. Endocrinology, *106:* 19–21, 1980.
11. Brodie, A. M. H., Marsh, D. A., and Brodie, H. J. Aromatase inhibitors. IV. Regression of hormone-dependent, mammary tumors in the rat with 4-acetoxyandrostene-3,17-dione. J. Steroid Biochem., *10:* 423–429, 1979.
12. Brodie, A. M. H., Marsh, D. A., Wu, J. T., and Brodie, H. J. Aromatase inhibitors and their use in controlling oestrogen-dependent processes. J. Steroid Biochem., *11:* 107–112, 1979.
13. Brodie, A. M. H., Romanoff, L. P., and Williams, K. I. H. Metabolism of the aromatase inhibitor 4-hydroxyandrostenedione by male rhesus monkeys. J. Steroid Biochem., *14:* 693–696, 1981.
14. Brodie, A. M. H., Schwarzel, W. C., and Brodie, H. J. Studies on the mechanism of estrogen biosynthesis in the rat ovary. I. J. Steroid Biochem., *7:* 787–793, 1976.
15. Brodie, A. M. H., Schwarzel, W. C., Shaikh, A. A., and Brodie, H. J. The effect of an aromatase inhibitor, 4-hydroxy-4-androstene-3,17-dione, on estrogen-dependent processes in reproduction and breast cancer. Endocrinology, *100:* 1684–1695, 1977.
16. Brodie, H. J., Kripalani, K. J., and Possanza, G. Studies on the mechanism of estrogen biosynthesis. VI. The stereochemistry of hydrogen elimination of C-2 during aromatization. J. Am. Chem. Soc., *91:* 1241–1242, 1969.
17. Brueggemeier, R. W., Floyd, E. E., and Counsell, R. E. Synthesis and biochemical evaluation of inhibitors of estrogen biosynthesis. J. Med. Chem., *21:* 1007–1011, 1978.
18. Covey, D. F., and Hood, W. F. Enzyme-generated intermediates derived from 4-androstene-3,6,17-dione cause a time-dependent decrease in human placental aromatase activity. Endocrinology, *108:* 1597–1599, 1981.
19. Covey, D. F., Hood, W. F., and Parikh, V. D. 10β-Propynyl-substituted steroids. Mechanism-based enzyme-activated irreversible inhibitors of estrogen biosynthesis. J. Biol. Chem., *256:* 1076–1079, 1981.
20. DeSombre, E. R., and Arbogast, L. Y. Effect of the antiestrogen C1628 in the growth of rat mammary tumors. Cancer Res., *34:* 1971–1976, 1974.
21. Goldenberg, I. S., Waters, N., Ravdin, R. S., Ansfield, F. J., and Segaloff, A. Androgenic therapy for advanced breast in women. J. Am. Med. Assoc., *223:* 1267, 1973.
22. Graves, P. E., and Salhanick, H. A. Seroselective inhibition of aromatase by enantiomers of aminoglutethimide. Endocrinology, *105:* 52–57, 1979.
23. Marcotte, P. A., and Robinson, C. H. Evaluation of a series of 10β-substituted estr-4-ene-3,17-dione derivatives as inhibitors/inactivators of human placental estrogen synthetase (aromatase). Fed. Proc., *40:* 1867, 1981.
24. Marsh, D. A., Romanoff, L. P., Williams, K. I. H., Brodie, H. J., and Brodie, A. M. H. Synthesis of deuterium and tritium labeled 4-hydroxyandrostene-3,17-dione, an aromatase inhibitor, and its metabolism *in vitro* and *in vivo* in the rat. Biochem. Pharmacol., *31:* 701–705, 1982.
25. Metcalf, B. W., Wright, C. L., Burkhart, J. P., and Johnston, J. O. Substrate-induced inactivation of aromatase by allenic and acetylenic steroids. J. Am. Chem. Soc., *103:* 3221–3222, 1981.
26. Miller, W. R., and Forrest, A. P. M. Oestradiol synthesis by a human breast carcinoma. Lancet, *2:* 866–868, 1974.
27. Perel, E., Wilkins, D., and Killinger, D. W. Inhibition of androgen aromatization and interconversion by 4-hydroxyandrostenedione in breast tissue. *In:* Abstracts of the 61st Annual Meeting of the Endocrine Society, Abstract 303, p. 247. Bethesda, Md.: Endocrine Society, 1979.
28. Samojlik, E., Veldhuis, J. D., Wells, S. A., and Santen, R. J. Preservation of androgen secretion during estrogen secretion during estrogen suppression with aminoglutethimide in the treatment of metastatic breast carcinoma. J. Clin. Invest., *65:* 602–612, 1980.
29. Santen, R. J., and Brodie, A. M. H. Suppression of estrogen production as treatment of breast carcinoma: pharmacologic and clinical studies with aromatase inhibitors. Clin. Oncol., in press, 1982.
30. Santen, R. J., and Samojlik, E. Medical adrenalectomy for treatment of metastatic breast carcinoma. *In:* W. McGuire (ed.), Breast Cancer, Vol. 3, pp. 79–114. New York: Plenum Publishing Corp., 1979.
31. Santen, R. J., Santner, S., Davis, B., Veldhuis, J., Samojlik, E., and Ruby, E. Aminoglutethimide inhibits extraglandular estrogen production in postmenopausal women with breast carcinoma. J. Clin. Endocrinol. Metab., *47:* 1257–1265, 1978.
32. Schwarzel, W. C., Kruggel, W., and Brodie, H. J. Studies of the mechanism of estrogen biosynthesis. VIII. The development of inhibitors of the enzyme system in human placenta. Endocrinology, *92:* 866–880, 1973.
33. Segaloff, A., Weeth, J. B., Meyer, K. K., Rongone, E. L., and Cunningham, M. E. G. Hormonal therapy in cancer of the breast. XIX. Effect of oral administration of Δ^1-testololactone on the clinical course and hormonal excretion. Cancer (Phila.), *15:* 633, 1962.
34. Siiteri, P. K., and Thompson, E. A. Studies of human placental aromatase. J. Steroid Biochem., *6:* 317–322, 1975.
35. Sjoerdsma, A. Suicide enzyme inhibitors as potential drugs. Clin. Pharmacol. Ther., *30:* 3–22, 1981.
36. Thompson, E. A., Jr., Hemsell, D., McDonald, P. C., and Siiteri, P. K. Inhibition of aromatization by steroidal drugs. J. Biol. Chem., *5:* 315, 1974.
37. Thompson, E. A., Jr., and Siiteri, P. K. Utilization of oxygen and reduced nicotinamide adenine dinucleotide phosphate by human placental microsomes during aromatization of androstenedione. J. Biol. Chem., *49:* 5364–5372, 1974.
38. Thompson, E. A., Jr., and Siiteri, P. K. The involvement of human placental microsomal cytochome P-450 in aromatization. J. Biol. Chem., *249:* 5373–5378, 1974.
39. Tsai-Morris, C. H., and Brodie, A. M. H. The effect of inhibiting proestrous estrogen on the LH surge and on ovulation. *In:* Society for the Study of Reproduction, 14th Meeting, Abstract 138, p. 91A. Champaign, Ill.: Society for the Study of Reproduction, 1981.
40. Uzgiris, V. I., Graves, P., and Salhanick, H. A. Liquid modification of corpus luteum mitochondrial cytochrome P-450 spectra and cholesterol monooxygenation: an assay of enzyme-specific inhibitors. Biochemistry, *16:* 593–600, 1977.
41. Uzgiris, V. I., Whipple, C. A., and Salhanick, H. A. Stereoselective inhibition of cholesterol side chain cleavage by enantiomers of aminoglutethimide. Endocrinology, *101:* 89–92, 1977.
42. Varela, R. M., and Dao, T. L. Estrogen synthesis and estradiol binding by human mammary tumors. Cancer Res., *38:* 2429–2433, 1978.