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COMMENTARY

AROMATASE INHIBITION AND ITS PHARMACOLOGIC IMPLICATIONS

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Inhibition of estrogen biosynthesis by selective blockade of the aromatase enzyme to reduce estrogen production could be an effective means of treating clinical problems associated with estrogen.

Aromatase is a ubiquitous enzyme occurring not only in reproductive tissues of the female but also in such diverse sites as testes, adipose tissue, and brain. Aromatase mediates the conversion of androgens to estrogen and is an enzyme complex involving an NADPH–cytochrome *c* reductase and a cytochrome P-450. The reaction is unique in the biosynthesis of steroids, as it involves conversion of ring A of the steroid molecule to an aromatic ring with the loss of the angular C-19 methyl group and *cis* elimination of the 1 β and 2 β hydrogens to yield estrogen and formic acid [1–4] (Fig. 1). Also, aromatization is the last in the series of steps in the biosynthetic progression from cholesterol to the estrogens. Therefore, blockade of this enzyme would not cause deprivation of other essential steroids. Our rationale for the clinical use of aromatase inhibitors is that compounds interacting with the aromatizing enzyme in all estrogen synthesizing tissues could provide both selective and effective inhibition of estrogen production. For example, aromatase inhibitors could be of value in treating gynecomastia, endometriosis, premature labor, idiopathic oligospermia, and endometrial and breast cancers.

Approximately 40,000 women die of breast cancer, and 100,000 new cases are diagnosed, each year in the U.S. About 60% of premenopausal and 75% of postmenopausal patients [5] have hormone-dependent carcinomas as identified by the presence of estrogen receptor (ER) in tumor biopsies. Deprivation of estrogen in such patients with metastatic disease results in tumor regression which is objectively quantifiable and may often be long-lasting.

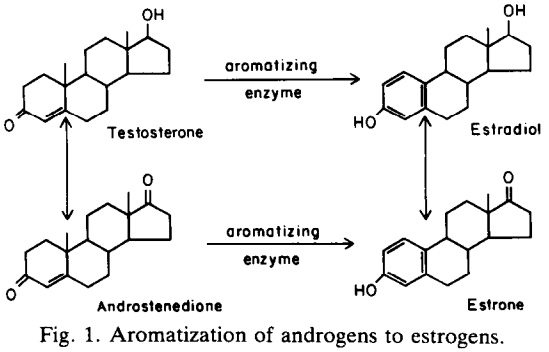

Fig. 1. Aromatization of androgens to estrogens.

Although estrogens are produced primarily by the ovaries in young women, after the menopause aromatization in extragonadal tissues [6, 7], such as fat and muscle, increases [8, 9] and is the main source of estrogens contributing to tumor growth. Some breast tumors are reported to aromatize [10–13] or produce estrogens locally from estrone sulfate [14, 15]. Even after hypophysectomy or adrenalectomy, patients may continue to produce estrogens in significant amounts [16]. Therefore, a systemic method of treatment with aromatase inhibitors could be a specific and effective means of lowering estrogen production from all sources and could be less traumatic than surgical methods. Cytotoxic agents have not been found to be highly effective or to cure breast cancer. Usually, estrogen-controlling treatments are much less toxic than chemotherapy and could be used to maximum effect in adjuvant therapy when the number of tumor cells is low. Also, they can be administered for long periods and may be useful in combination therapy regimens. Recent evidence indicates that patients with estrogen receptor positive tumors have a better prognosis and longer disease-free intervals. Thus, development of new estrogen-controlling strategies is appropriate.

Endometrial cancer is highly correlated with obesity [17]. Not only does this increase the risk of surgery for these patients, but also the production of peripherally formed estrogen is enhanced [18]. Treatment with aromatase inhibitors may be beneficial for this type of cancer also.

Endometriosis occurs more frequently than any other gynecological disorder except uterine fibromyomata [19]. This condition can be painful and debilitating due to the growth of ectopic endometrial tissue which undergoes cyclic changes. About one-third of patients with endometriosis are infertile. Surgery is often ineffective and is unsuitable for women of reproductive age. A reversible systemic approach, which would affect endometrial tissue irrespective of its location, by inhibiting ovarian estrogen production could be more acceptable and effective. Danazol, currently being used, is not well tolerated and often inadequate. Aromatase inhibitors may be useful alternatives.

In recent studies, treatment of rhesus monkeys

with an aromatase inhibitor for a few days during late pregnancy was found to inhibit estrogen production and delay the onset of labor contractions initiated by fetal instrumentation [20]. If these preliminary results can be substantiated, this approach involving short-term treatment with aromatase inhibitors could be useful in treating premature labor, a commonly occurring problem in obstetrics.

Vigersky and Glass* have reported that idiopathic oligospermia is treated effectively by inhibiting estradiol production and thereby increasing the testosterone/estrogen ratio. Sperm density and total sperm count improved in eleven of the fifteen patients treated. No further benefit was observed by the addition of the antiestrogen tamoxifen to block any residual estrogen effect. Thus, a role for aromatase inhibitors is indicated for this condition.

Although the oral contraceptive pill has made a tremendous impact on world population growth, there is still a need for long-acting contraceptives especially for use in countries where health care facilities and supplies are limited. Despite recent efforts, depoprovera has not been approved for use in this country. Aromatase inhibitors could be used to block ovulation by inhibiting preovulatory estrogen but without inhibiting basal estrogen secretion. Our evidence in the rat suggests this may be a feasible approach.†

In addition to their clinical application, aromatase inhibitors are valuable research tools [21, 22] and were used recently to investigate the role of estrogens in the maturation of the ovarian follicle. The studies indicate that estrogens are important in the early stages of follicular development for induction of LH receptors on the granulosa cells [23] and for acquisition of meiotic competence by the oocyte [24]. However, the later stages of maturation such as polar body formation in the oocyte and rupture of the follicle during ovulation appear to be independent of estrogens [25]. These findings are consistent with our observations that aromatase inhibitor treatment during the early part of the cycle rather than near to the time of the LH surge is more effective in preventing ovulation in the rat.†

Inhibition of aromatase

Selective inhibition of aromatase may be achieved by compounds which interfere with androgen aromatization on binding to the enzyme. Such compounds produce type I high-spin spectra [26]. In 1973, our group published the first report on inhibitors of this type as part of our program to develop aromatase inhibitors for application to breast cancer and contraception [27].

Inhibition of estrogen biosynthesis may also be achieved by compounds which interfere with steroid hydroxylations by binding to cytochrome P-450. However, these inhibitors would be less specific in their actions. Aminoglutethimide (AG) is an inhibitor of this type and was demonstrated to inhibit aromatase by Chakraborty *et al.* [28] and by Thompson and Siiteri [26]. By competitive inhibition of cytochrome P-450, AG interferes with desmolase, the enzyme mediating the cholesterol side-chain cleavage, 11-hydroxylase, 21-hydroxylase, and 18-hydroxylase as well as aromatase [29, 30]. AG was found to be a more potent inhibitor of aromatase than of the other steroid hydroxylases except for 18-hydroxylase for which it is slighly more potent. AG is a racemic mixture of D and L stereoisomers. However, the D-isomer is 30-fold more potent than the L form for aromatase inhibition, whereas the L-enantomer is 15-fold more potent than the D form with respect to inhibition of cholesterol side-chain cleavage [29, 31]. This compound was first introduced as an anticonvulsant, but was observed to cause adrenal insufficiency which led to restriction of its use. Subsequently, because of this action, it was used to produce medical adrenalectomy. That aromatase inhibitors may be effective clinically was later demonstrated in studies of breast cancer patients treated with AG [32]. AG has now been shown to inhibit extragonadal estrogen production in postmenopausal breast cancer patients and to produce objective disease remission to the same extent as surgical adrenalectomy [33, 34], establishing it as a useful agent for breast cancer treatment. Several specific aromatase inhibitors are more potent, as discussed below, and may be effective in premenopausal as well as postmenopausal patients. These compounds may have milder side effects than AG, and corticoid replacement therapy is not required.

Evaluation of compounds as type I aromatase inhibitors in vitro

Over the past several years, we have evaluated a large number of compounds as type I inhibitors of aromatase [27, 35]. Candidate inhibitors were tested *in vitro* by comparing the extent of aromatization in incubations of microsomes from aromatase containing tissues. The conversion of androstenedione to estrogen by the microsomal preparation can easily be estimated by measuring the loss of tritium from the C-1 β and C-2 β positions [2] during aromatization of [1,2^{3}H(70% β)]androstenedione. The tritium released as ^3H_2O is measured in the incubation medium after extraction of steroids by organic solvent [26]. First, human placental microsomes were used as the source of aromatase [28] and later a highly active microsomal preparation was developed from ovaries of rats stimulated with pregnant mares' serum gonadotropin (PMSG) [36]. Our studies of the two microsomal preparations suggest that subtle differences exist between aromatases from the two sources. The ovarian microsomal preparation was considered more appropriate for predicting *in vivo* activity in the rat.

The inhibitors with greatest activity in both systems are 4-hydroxyandrostene-3,17-dione (4-OHA) [37], 4-acetoxyandrostene-3,17-dione (4-acetoxyA) [38] and 1,4,6-androstatriene-3,17-dione (ATD) [39]. 4-OHA has been shown to have activity against human ovarian aromatase in granulosa cell cultures [40]. All show Lineweaver–Burk plots typical of competitive inhibition (Fig. 2) [37] which occurs

* R. A. Vigersky and A. R. Glass, *Abstracts of the Sixty-fifth Annual Meeting of the Endocrine Society*, San Antonio, 726 (1983).

† C-H. Tsai-Morris and A. M. H. Brodie, *Society for the Study for Reproduction*, Abstr. 223 (1982).

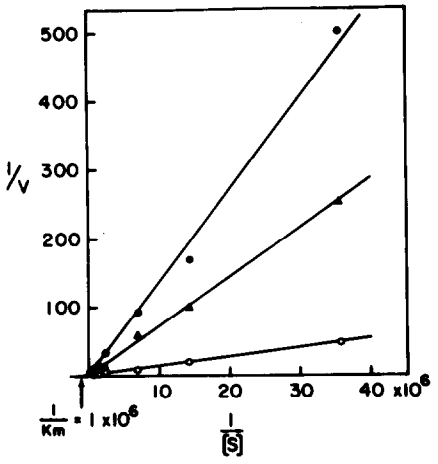

Fig. 2. Lineweaver–Burk plots of 4-OHA inhibition in rat ovarian microsomes. Aromatization was determined from tritium released and converted to ^3H_2O during incubation of [1,2-^{3}H(70% β)]androstenedione and 4-OHA for 30 min at 37° with microsomes from ovaries of PMSG-primed rats and the NADPH-generating system. $K_m = 1 \times 10^{-6}$ M. Key: (○——○) no inhibitor; (△——△) 1.3 μM and (●——●) 2.6 μM 4-OHA present (from Brodie *et al.* [37]).

rapidly in the presence of both substrate (androstenedione) and inhibitor. 4-OHA, 4-acetoxy-A [41, 42], ATD and A-trione [39, 43] also cause slower time-dependent loss of enzyme activity which follows pseudo first-order kinetics in microsomes preincubated in the absence of substrate, but in the presence of NADPH (Fig. 3). There was no loss of activity without added cofactors. Although 4-OHA caused the most rapid inactivation, the loss of activity with all three compounds was 10-fold slower in rat ovarian microsomes compared with the placental system. Aromatase activity was not regained after washed microsomes preincubated with 4-OHA had been allowed to stand for 18 hr at 0° followed by charcoal treatment and exhaustive washing to remove any residual inhibitor. These findings suggest that 4-OHA causes long-term inactivation (or irreversible inhibition) of aromatase.

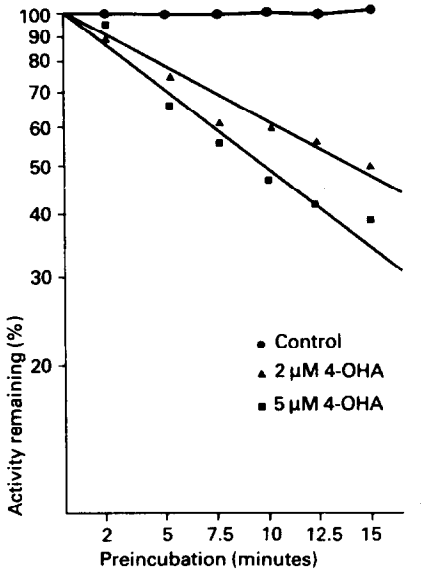

Fig. 3. Inactivation of human placental aromatase by 4-OHA. Human placental microsomes were preincubated with 1 mM NADPH and 4-OHA at 37°. After removal of 4-OHA with charcoal, aromatase activity was estimated from tritiated water (^3H_2O) released during incubation with [1,2-^{3}H]androstenedione for 30 min (from Brodie *et al.* [41]).

Aromatase inactivation has also been reported to occur with the 10-propargyl analog of androstenedione [10-(2-propynl)estr-4-ene-3,17-dione] in the placental system [44–46] and was confirmed by us in both the placental and rat ovarian systems (unpublished observations). This compound was designed to inactivate aromatase by binding covalently to the enzyme. The 10-propargyl analog and other inhibitors synthesized by these investigators are allenes which probably lead to allene oxide intermediates via oxygen insertion by aromatase. The intermediate would alkylate either the prosthetic heme or surrounding enzymic protein causing inactivation of the enzyme [47, 48]. Although the precise mechanisms by which 4-OHA, 4-acetoxyA and ATD inactivate aromatase are unknown at present, their kinetics are similar to those of the 10-propargyl analog and suggest that they are k_{cat} or suicide inhibitors. Inhibitors of this type have been successfully developed as drugs for other enzymes. Since they bind to the active site of the enzyme, they are quite specific and have long-lasting effects *in vivo* due to inactivation of the enzyme [49].

Aromatase is inhibited approximately four times faster by 4-OHA than the 10-propargyl in *in vitro* preparations. On the other hand, the latter has a K_i of 4.5 nM with placental microsomes and has two to three times greater affinity for the active site of the enzyme than 4-OHA (K_i 10.2 nM) [47]. 16α-Bromoandrogens reported by Bellino and colleagues [50] and 7p-amino-thiophenylandrostenedione synthesized by Counsell and co-workers [51] are also potent aromatase inhibitors but have not been studied in detail *in vivo*. Testololactone, a compound used for breast cancer treatment for over 20 years [52], and subsequently reported to be an aromatase inhibitor [26] was found recently to cause aromatase inactivation, probably by virtue of the C-1 double bond [47]. However, similar to AG (K_i 770 μM), it has weak activity (K_i 750 μM, $T_{1/2}$ 21 min) in placental microsomes, compared to the above [47].

In vivo *actions of aromatase inhibitors in animal models*

Inhibition of ovarian estrogen secretion. 4-OHA, 4-acetoxyA and ATD were evaluated further to determine whether they had activity against ovarian estrogen biosynthesis *in vivo*. To overcome the normal cyclicity of the rat and maintain a constant estrogen secretion, aromatase activity was stimulated by first priming the animals with PMSG over 11 days [36]. On day 12, the animals, were injected with 4-OHA or ATD. At various times after injection, blood was first collected from the ovarian vein, and then microsomes were prepared from the ovaries. Aromatase activity in the ovarian microsomes was

reduced to 20% of the initial value within 8 hr after injection and remained low for 24 hr following injection of ATD and for 48 hr following injection of 4-OHA. Estrogen concentrations in the ovarian vein samples were also reduced by inhibitor treatment and remained low for about the same length of time as aromatase activity. These findings suggest that enzyme inactivation is occurring in the ovary *in vivo* with both ATD [40] and 4-OHA [42], since activity could not be increased by procedures designed to remove unbound 4-OHA.

In normal cycling rats, ovarian aromatase activity and estrogen secretion during proestrus are also inhibited markedly (at least 85%) 3 hr after an injection of 4-OHA, ATD and 4-acetoxy [53]. A significant decrease in ovarian aromatase activity of about 70% is reported to occur in mice primed with 20 I.U. PMG and treated with the 10-propargyl compound from silastic implants delivering about 1 mg/kg/day [54].

Antitumor activity of aromatase inhibitors in the 7,12-dimethylbenz[a]anthracene (DMBA)-induced mammary carcinoma model. The DMBA-induced carcinoma model has been used extensively to study hormone-dependent mammary tumors. Although other hormones may be involved, the tumors are dependent on ovarian production of estrogen. Mammary tumors are induced in the female rat aged 50–55 days by a single feeding of the carcinogen (DMBA), [55]). After approximately 6–8 weeks, multiple mammary tumors develop of which about 80–90% are hormone dependent.

Groups of rats treated with aromatase inhibitors were matched to controls as closely as possible for the number of animals and tumors and for total tumor volume. Tumors are measured with calipers and their volumes calculated [56]. Treatment with 4-OHA, 4-acetoxyA or ATD either administered as twice daily injections (50 mg/kg/day) or from silastic implants caused marked tumor regression [37–39]. After 4 weeks of 4-OHA treatment, the total tumor volume in the experimental group was reduced by about 80% of the initial volume. At the end of 4 weeks of treatment, ovarian aromatase activity and estrogen secretion were both markedly inhibited compared to controls (Fig. 4) [53, 57]. Also, physiological doses (0.2 μg/rat) of estradiol administered concomitantly with 4-OHA for 4 weeks prevented tumor regression. These results suggest that tumor regression induced by 4-OHA and the above inhibitors is a result of inhibition of ovarian estrogen production.

In other studies with 4-OHA, when the inhibitor was given twice daily for 1 week, tumor regression continued for a further 2 weeks without treatment. Furthermore, tumor suppression induced by twice daily injections for 1 week could be maintained for at least 20 weeks by twice weekly injections [58]. Thus, 4-OHA appears to have long-lasting effects *in vivo* which may be related to inactivation of ovarian aromatase.

Aromatase inhibitors 4-OHA and 4-acetoxyA caused greater tumor regression in the DMBA-induced tumor model than tamoxifen [59]. The com-

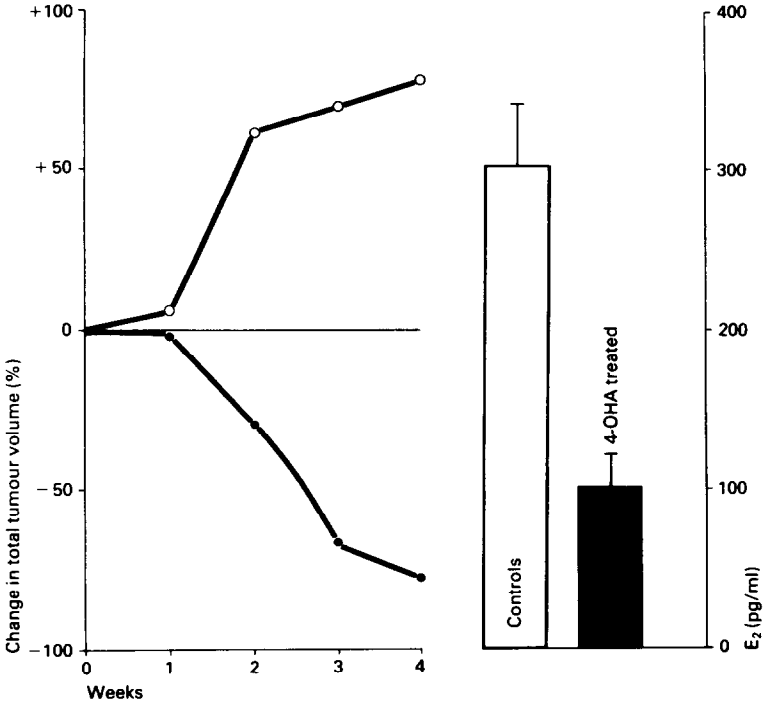

Fig. 4. Effect of 4-OHA on DMBA-induced, hormone-dependent mammary tumors of the rat. Key: (●) percentage change in total volume of thirteen tumors on six rats injected with 4-OHA (50 mg/kg/day) twice daily for 4 weeks; (○) tumors on five control rats injected twice daily with vehicle. At the end of treatment blood was collected from each rat by ovarian vein cannulation for estradiol (E_2) assay; controls were sampled during diestrus (from Brodie *et al.* [57]).

bined effect of these agents, which block estrogen action as well as secretion, did not result in greater tumor regression than with aromatase inhibitors alone. Tamoxifen is known to be a partial estrogen agonist in the rat, and it is likely that this property is responsible for retarding the full effect of the aromatase inhibitors when used in combination. Further evidence of the agonistic action of tamoxifen in the rat was seen when animals with mammary tumors were ovariectomized and administered tamoxifen for 4 weeks. Tumor regression due to ovariectomy was retarded by tamoxifen treatment but not by 4-OHA (50 mg/kg/day) [58].

Other in vivo *actions of steroidal aromatase inhibitors.* Type I inhibitors are all steroids similar to androstenedione. For these compounds to be useful clinically, it is important to determine whether there are other activities *in vivo.* Bioassays indicated no estrogenic, progestational or antihormonal effects. However, 4-OHA, 4-acetoxy and ATD have less than 1% the androgenic activity of testosterone [37–39].

In mammary tumor studies, it was observed that, at the end of 4 weeks of treatment with 4-OHA, gonadotropin concentrations were not increased. Although estrogen secretion was consistently reduced by 4-OHA, gonadotropic levels were similar to basal levels for control rats during diestrus. Furthermore, 4-OHA treatment was found to prevent the increases in LH and FSH that occur after ovariectomy [60], indicating direct action of the compound on the pituitary-hypothalamus. We recently demonstrated that the coadministration of the antiandrogen flutamide but not the antiestrogen enclomiphene counteracts the inhibition of LH and FSH by 4-OHA and found similar results with dihydrotestosterone [61]. This suggests that the action of 4-OHA on the pituitary may be mediated by its weak androgenicity. Since ovarian aromatase activity is under the regulation of gonadotropins, new enzyme synthesis would not occur following aromatase inactivation by 4-OHA. Overall, this effect would contribute to maintaining inhibition of estrogen biosynthesis in the ovary. In contrast, gonadotropin levels were found to be increased in rats after 4 weeks of treatment with AG. Although ovarian aromatase and estrogen secretion were inhibited 3 hr after injection of AG, after 4 weeks, estradiol levels were not consistently suppressed and the total volume of mammary tumors was increased [62].

Although AG is effective in reducing postmenopausal estrogen levels [33], Santen *et al.* [63] observed that the mid-cycle surge of estrogen was not consistently suppressed in premenopausal women. 4-OHA might be more effective in this regard.

Inhibition of peripheral aromatization. Since extraovarian aromatization is an important source of estrogens in the postmenopausal breast cancer patients, the effects of 4-OHA and 4-acetoxyA on peripheral aromatase were studied by measuring the conversion of androstenedione to estrone during constant infusion of [7-^{3}H]androstenedione and [4-^{14}C]estrone. Male rhesus monkeys were used since most of the circulating estrogen in the male is of extragonadal origin. Each animal was infused under control conditions and during inhibitor treatment. 4-OHA was injected 18 hr and then 3 hr before the infusion was started. Peripheral aromatization was found to be undetectable in three of the four monkeys treated with 4-OHA and markedly reduced in the fourth animal. Treatment of two monkeys with 4-acetoxyA implants was also effective in reducing peripheral aromatization (Table 1) [64].

4-OHA is cleared rapidly from the circulation. The metabolic clearance rates of 4-OHA in two rhesus monkeys were 1730 liters of blood/day and 935 liters of blood/day [65]. However, when control infusion experiments were performed 1 week after 4-OHA treatment, peripheral aromatization values were significantly below the mean value previously reported for these animals [66], indicating sustained action of 4-OHA. This may be due to enzyme inactivation or to subcutaneous injection of 4-OHA suspension which may create a depot effect.

Clinical studies with aromatase inhibitors. As already discussed, aminoglutethimide has now been used in a number of clinical trials and investigations. These have been reviewed by Santen and colleagues [33, 34]. AG is effective in postmenopausal breast cancer patients and remission occurs in 40% of unselected patients. The compound is active in some patients who have relapsed from tamoxifen, indicating it can be used in addition to tamoxifen as well as an alternative treatment. Although somnolence is a side effect of AG, recent studies with low dose AG (500 mg/day) indicate it to be as effective as the higher dose (1 g/day) and is better tolerated [67].

Because of its greater potency *in vitro*, high efficacy in animal models, and ease of synthesis, 4-OHA was selected for the first clinical trial with

Table 1. Effect of 4-OHA and 4-acetoxyA on peripheral aromatization in rhesus monkeys*

	Monkey	$[\rho]^{A,E_1}_{BB}$†	CR^{A,E_2}_{BB}†
1	Control	ND§	0.011
	After 4-OHA	ND	ND
2	Control	0.0061	0.0034
	After 4-OHA	0.0026	ND
3‖	Control	0.0103	0.0083
	After 4-OHA	0.0013	0.0019
4‖	Control	0.011	0.015
	After 4-OHA	0.0007	ND
1	Control	0.022	0.018
	After 4-acetoxyA	0.0007	0.0008
2	Control	0.0071	0.0053
	After 4-acetoxyA	0.011	0.013

* Male rhesus monkeys were infused with [7-^{3}H]androstenedione and [4-^{14}C]estrone before and during treatment with 4-OHA (50 mg/kg) or 4-acetoxyA (700 mg each from silastic implants and injections).

† The fraction of infused [7-^{3}H]androstenedione measured as [^{3}H]estrone in blood.

‡ Conversion ratio of androstenedione to estradiol.

§ Not detectable.

‖ Monkeys 3 and 4 control data were obtained 1 week after treatment.

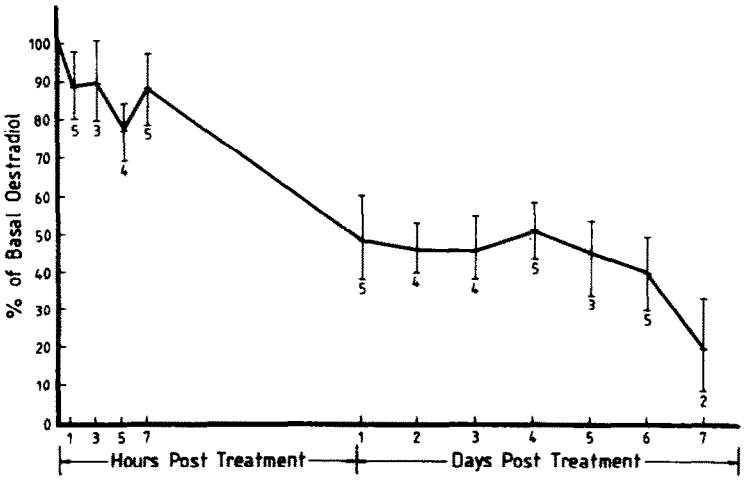

Fig. 5. Effect of a single dose of 500 mg 4-OHA on plasma estradiol in five patients with breast cancer. The basal estradiol for each patient was calculated by averaging seven pre-treatment values, and the post-treatment results were calculated as the percentage of that baseline. The mean of the percentage of baseline for the five patients is shown. Basal samples were obtained at four identical time points as the samples obtained 1 day post-treatment. Results are shown as mean ± S.D. (from Coombes *et al.* [68]).

a specific aromatase inhibitor. A small number of postmenopausal patients with advanced metastatic breast cancer have now been treated with this compound. In the initial report, eleven patients with histologically proven advanced progressive disease with measurable lesions were treated with intramuscular injections of 500 mg 4-OHA for 3–20 weeks [68]. None had received endocrine therapy or chemotherapy within 4 weeks of starting 4-OHA treatment. Eight patients received one injection per week and three patients received one injection every 2 weeks. No adverse effects of 4-OHA were encountered except for pain at the site of injection in some patients. Tumor estrogen receptor concentrations were unknown for six patients, and five patients had estrogen receptor positive tumors. The mean serum estradiol concentration of five patients was decreased significantly 24 hr after treatment and was 20% of the pretreatment level after 7 days (Fig. 5). Partial or complete remission of measurable lesions occurred in four patients. In one patient, lytic metastases of ribs and pelvis showed resclerosis and, in another, severe bone pain was alleviated. Three of the responders had responded previously to endocrine therapy but had relapsed, and the fourth patient had estrogen receptor positive tumors. Seven other patients did not respond to 4-OHA therapy although the disease was stabilized in one. However, none of these, except one, had responded to previous endocrine therapy. Although the optimal dose of 4-OHA has not yet been established and only a small number of patients have been treated to date, the results suggest that this aromatase inhibitor may be beneficial to women with hormone-responsive metastatic breast cancer. It remains to be determined whether this compound or similar aromatase inhibitors can be effective in other estrogen-related conditions.

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