

Effect of Treatment with 4-Hydroxyandrostenedione on the Peripheral Conversion of Androstenedione to Estrone and *in Vitro* Tumor Aromatase Activity in Postmenopausal Women with Breast Cancer¹

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ABSTRACT

The effect of treatment with the aromatase inhibitor, 4-hydroxyandrostenedione (4-OHA) on the peripheral conversion of androstenedione to estrone has been examined in eight postmenopausal women with advanced breast cancer. Before treatment conversion of androstenedione to estrone ($[\rho]_{BB}^{AE1}$) ranged from 0.81 to 3.7% and was almost completely inhibited after treatment with 4-OHA (two doses of 500 mg i.m. with an interval of 12 days between doses). Transfer constants were also measured by the urinary method ($[\rho]_{BU}^{AE1}$) for some subjects and decreased from $2.3 \pm 0.52\%$ to $0.24 \pm 0.11\%$ after treatment, a mean reduction of 90%. Mean plasma concentration of estradiol (37.4 ± 16.6 pmol/liter) and estrone (99.0 ± 32.2 pmol/liter) decreased significantly ($P < 0.01$) to 15.7 ± 4.6 pmol/liter and 52.4 ± 8.9 pmol/liter, respectively, after treatment. Aromatase and DNA polymerase α (a marker of cell proliferation) activities were measured in seven samples of breast tumor tissue obtained before and after treatment. For three samples there was a marked ($67 \pm 17\%$) decrease in tumor aromatase activity after treatment, for two, little change occurred, while tumor aromatase activity in the other two samples appeared to be resistant to the effect of 4-OHA. The correlation between tumor aromatase and DNA polymerase α activities ($r = 0.45$) failed to reach a significant level.

INTRODUCTION

Estrogen formation in postmenopausal women results almost exclusively from the peripheral conversion of androstenedione to estrone (1, 2). This reaction is mediated by the aromatase enzyme complex which is present in adipose, muscle, and some, but not all, breast tumor tissues (3, 4). As many breast tumors are hormone dependent the use of inhibitors of aromatase activity can provide an acceptable alternative to surgical ablative procedures. Santen *et al.* (5) have previously shown that the use of AG³ for the treatment of breast cancer effectively inhibits peripheral conversion of androstenedione to estrone. However, the use of AG can be associated with a number of adverse side-effects and requires the coadministration of hydrocortisone to suppress adrenocorticotrophic hormone secretion, which increases due to the inhibition of enzymes involved in the synthesis of cortisol.

An important development in the use of aromatase inhibitors was the synthesis of 4-OHA which is a specific inhibitor of aromatase activity (6). 4-OHA has been successfully used for the treatment of postmenopausal women with breast cancer (7), but the extent to which this steroid inhibits peripheral aromatase activity and tumor aromatase activity has not yet been determined.

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³ The abbreviations used are: AG, aminoglutethimide; 4-OHA, 4-hydroxyandrostenedione; MCR, metabolic clearance rate; SHBG, sex-hormone binding globulin; GC-MS, gas chromatography-mass spectrometry.

In the present study we have therefore examined the effect of treatment on the peripheral conversion of androstenedione to estrone in eight postmenopausal women with breast disease. Plasma estrone and estradiol concentrations, together with the MCR for androstenedione and estrone were also measured before and after treatment. Samples of breast tumor tissue were also obtained before and after treatment to examine the effect of 4-OHA on tumor aromatase activity, as measured *in vitro*. The activity of DNA polymerase α , a marker of cellular proliferative activity, was also measured. If local synthesis of estrogen within tumors exerts a biological effect, then any decrease in aromatase activity might be associated with a decrease in cell proliferation.

MATERIALS AND METHODS

Patients. Eight postmenopausal women with breast disease took part in this study after giving their informed consent. Details of patients age, number of years postmenopause, body mass index, and tumor size are shown in Table 1. Seven of the women had advanced breast cancer (Stage III-IV) while one was found to have a cystosarcoma phylloides. Patients were investigated before undergoing surgery to obtain an initial biopsy specimen and again about 2 weeks later before surgery for removal of their tumors.

Transfer Constants. To measure transfer constants for the conversion of androstenedione to estrone, isotopically labeled [1,2,6,7-³H]androstenedione (85 Ci/mmol; Amersham International, UK) and [4-¹⁴C]estrone (56 mCi/mmol; Amersham International) were infused for a 12-h period overnight. The ³H-androstenedione used is specifically labeled in the 1 α and 2 α positions of the steroid nucleus which are not involved in the aromatization process (8). Purity of isotopically labeled steroids was checked before infusion by paper chromatography using light petroleum:toluene:methanol:water (33:17:40:10 by volume) as solvents. Steroids were infused in 5% ethanolic saline containing 4% human serum albumin at a rate of 12 μ Ci/h for [³H]androstenedione and 0.6 μ Ci/h for [¹⁴C]estrone. At about 11.5 h and 12.0 h of tracer infusion blood samples (50 ml) were taken, and after removal of the red blood cells plasma was stored at -20°C until assayed. Samples of breast tumor tissue were obtained at surgery (when the second sample of blood was taken) and sections were taken for histology and receptor analysis with remaining tissue frozen in liquid nitrogen and stored at -70°C until processing.

Patients received two doses of 4-OHA (500 mg, i.m.) at an interval of 12 days (Fig. 1). 4-OHA was provided by Ciba-Geigy Pharmaceuticals as a sterile microcrystalline formulation. The powder was suspended in physiological saline (125 mg/ml) immediately prior to i.m. injection. Infusion of isotopically labeled steroid was repeated overnight 24 h after the second dose of 4-OHA was injected, prior to surgery for tumor removal. For three patients, in addition to collecting blood to measure transfer constants by the blood method, urine was also collected for 3 days, before and after treatment, to enable transfer constants to be measured by the urinary method.

Transfer constants ($[\rho]_{BB}^{AE1}$ and $[\rho]_{BU}^{AE1}$), together with the MCR values for androstenedione and estrone were measured as previously described (2, 9). Briefly, isotopically labeled estrone was extracted from plasma with diethyl ether, while urine was initially treated with β -glucuronidase enzyme preparation to liberate conjugated estrone, before extraction.

Table 1 Clinical details

Age (years)	Years post-menopause	BMI ^a	Tumor size (cm)
69.3 ± 9.1	18.6 ± 9.9	25.5 ± 5.6	6.7 ± 2.7

^a BMI, body mass index (weight/height²).

PROTOCOL

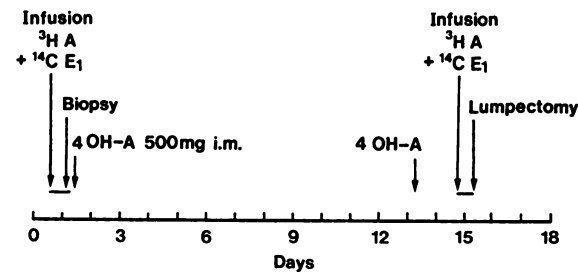


Fig. 1. Study protocol. [³H]Androstenedione (³HA) and [¹⁴C]estrone (¹⁴C E1) were infused overnight before patients underwent surgery to obtain a biopsy sample. Postoperatively patients received 4-hydroxyandrostenedione (4OH-A, 500 mg, i.m.) with a further dose being given 12 days later. One day after receiving the second dose of 4OH-A the isotopic infusion was repeated prior to surgery for tumor removal.

The residue, containing estrone, obtained after evaporation of the diethyl ether was purified by Lipidex 5000 column chromatography (Packard, Reading, UK) and three further chromatographic purifications using thin-layer chromatography. Transfer constants were calculated by comparing the ratio of [¹⁴C]estrone to [³H]androstenedione infused to the ¹⁴C:³H ratio of estrone isolated from blood or urine. [³H]Androstenedione was extracted from plasma and purified by a similar procedure and with [¹⁴C]estrone used to calculate the MCR values from the rate of isotopic infusion divided by the concentration of [³H]-androstenedione or [¹⁴C]estrone in plasma (10). [4-¹⁴C]Androstenedione (Amersham International) together with unlabeled androstenedione (100 µg; Sigma, UK) and estrone (100 µg; Sigma, UK) were added to monitor procedural losses and act as carriers (11). Overall recoveries of [4-¹⁴C]androstenedione and unlabeled estrone from plasma were 58.6 ± 11.8% and 58.5 ± 9.2, respectively.

Plasma concentrations of estrone and estradiol were measured by radioimmunoassay (12) and SHBG by a radioimmunometric method (13). Cross-reaction of 4-OHA with the antisera used for the measurement of estrone and estradiol was less than 0.001%.

In addition to obtaining blood samples from patients in whom transfer constants were measured, estrone and estradiol levels were measured in blood obtained from another woman with breast cancer, before and after treatment, for whom it was not possible to complete the infusions of isotopically labeled steroids.

In vitro tumor aromatase activity was measured using a tritiated water release technique (14). DNA polymerase α activity was measured according to a method previously described (15).

RESULTS

Treatment with 4-OHA resulted in the almost complete inhibition of the peripheral conversion of androstenedione to estrone (Table 2). For transfer constants measured in blood ([ρ]_{BB}^{E1}) conversion of androstenedione to estrone ranged from 0.81 to 3.78%. After treatment no [³H]estrone was detectable in blood for four of the eight patients and for the remaining subjects was greatly reduced. Transfer constants were also measured by the urinary method for three subjects (Table 2). By this method conversion of androstenedione to estrone decreased from 2.37 ± 0.52% to 0.24 to 0.11%, a mean reduction of 90%.

In spite of the almost complete inhibition of peripheral aromatase activity, significant concentrations of estradiol and estrone were still detectable in plasma after treatment with 4-

Table 2 Transfer constants for the conversion of androstenedione to estrone [ρ]^{E1}, measured by blood (BB) and urinary (BU) methods before and after treatment with 4-OHA

Subject	[ρ] _{BB} ^{E1} (%)		[ρ] _{BU} ^{E1} (%)	
	Before	After	Before	After
1	1.22	ND ^a	2.1	0.23
2	1.07	0.28		
3	3.78	ND		
4	2.55	0.28	3.09	0.14
5	1.54	ND		
6	0.81	0.43		
7	1.13	ND	1.90	0.35
8	2.3	0.26		

^a ND, no [³H]E1 detectable.

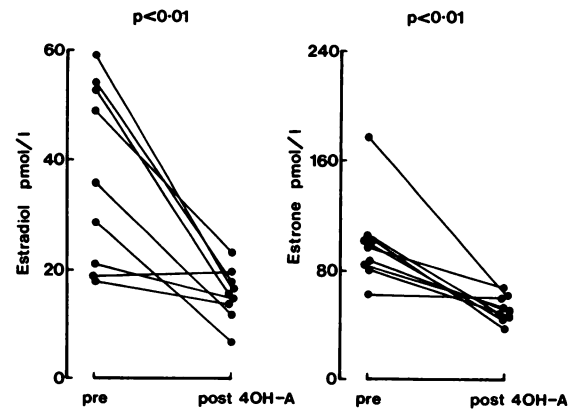


Fig. 2. Plasma concentrations of estradiol and estrone before and after treatment with 4-hydroxyandrostenedione (4OH-A).

Table 3 Metabolic clearance rates for androstenedione (MCR-A) and estrone (MCR-E1) before and after treatment with 4-OHA

Subject	MCR-A (liters/24 h)		MCR-E1 (liters/24 h)	
	Before	After	Before	After
1	1247	937	1103	1172
2	1513	1257	1389	2569
3	1309	1485	1415	1428
4	1339	1562	1328	1494
5	355	1211	463	1588
6	1365	1726	884	2123
7	1350	1261	1186	2174
8	1488	1622	2372	2093
Mean ± SD	1246 ± 371	1383 ± 261	1268 ± 546	1830 ± 476
	NS		P < 0.05	

OHA (Fig. 2). Overall mean concentrations of estradiol and estrone of 37.4 ± 16.6 pmol/liter and 99.0 ± 32.2 pmol/liter were both significantly reduced (P < 0.01) to 15.7 ± 4.6 pmol/liter and 52.4 ± 8.9 pmol/liter, respectively. This represents a mean decrease of 58 and 47%, respectively, for estradiol and estrone concentrations. The mean concentration of SHBG measured in plasma before (88 ± 62 nmol/liter) and after treatment (88 ± nmol/liter) did not differ significantly.

The MCR values for androstenedione and estrone measured before and after treatment are shown in Table 3. The MCR values for androstenedione showed no significant change overall after treatment, although for one subject a marked increase did occur. A similar increase in MCR for estrone was also seen for this subject and overall there was a small but significant (P < 0.05) increase in the MCR estrone after treatment.

The effect of treatment with 4-OHA on *in vitro* tumor aromatase and DNA polymerase α activities are shown in Fig. 3. In three samples of tumor obtained after treatment there was a marked (67 ± 17%) decrease in tumor aromatase activity, as measured *in vitro*. For two other subjects little change in *in vitro* tumor aromatase activity was detected while for the re-

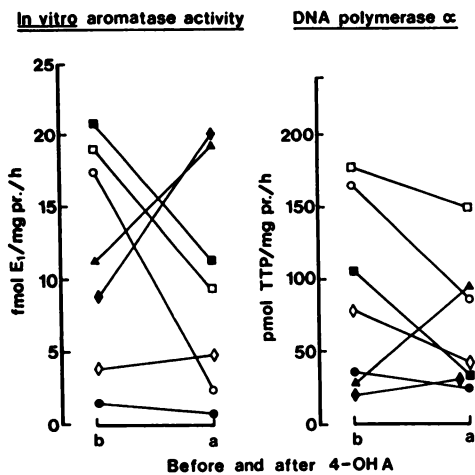


Fig. 3. *In vitro* tumor aromatase and DNA polymerase α activities in breast tumor samples obtained before and after treatment with 4-OHA.

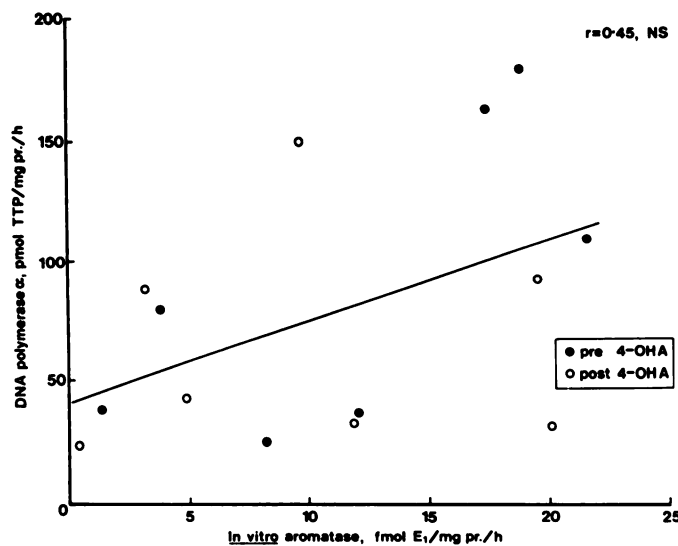


Fig. 4. Correlation between DNA polymerase α and aromatase activities in breast tumor samples obtained before and after treatment with 4-OHA.

maintaining subjects, aromatase activity, as measured *in vitro*, appeared to be resistant to the inhibitory effect of 4-OHA and increased compared with pretreatment values. For one of these subjects DNA polymerase α activity was also increased while for other subjects activity decreased (3 of 7) or showed little change. Tumour aromatase and DNA polymerase α activities were not measured for the subject found to have a cytosarcoma phylloides. Overall there was no significant reduction in tumor aromatase or DNA polymerase α activities. The correlation between DNA polymerase α activity and tumor aromatase activity measured *in vitro* ($r = 0.45$, NS) failed to reach a significant level (Fig. 4).

DISCUSSION

4-Hydroxyandrostenedione has been successfully used in the treatment of breast cancer (7). The results from the present study show that peripheral conversion of androstenedione to estrone is greatly reduced after treatment with 4-OHA. The reduction after treatment with 4-OHA is similar to that found to occur after treatment with AG (5) or in the rhesus monkey after administration of 4-OHA (16).

In spite of the almost complete inhibition of the peripheral

conversion of androstenedione to estrone by 4-OHA, significant plasma concentrations of estradiol and estrone remained detectable. While some previous reports only noted a significant reduction in estradiol concentrations after treatment with 4-OHA (17), Dowsett *et al.* have recently shown that circulating estrone levels also decrease when measured using a GC-MS method (18). The reduction in circulating estrone concentrations reported in the present study (47%) is therefore in good agreement with the reduction (60%) detected using a GC-MS method. The decrease in circulating plasma estradiol concentrations of almost 60% is also in good agreement with previous investigations (18, 19). The detection of significant concentrations of estrone after treatment, by GC-MS and the specific radioimmunoassay used in the present study, at a time when peripheral conversion of androstenedione is almost completely inhibited, requires explanation. The most likely origin of estrone detectable after treatment with 4-OHA for less than 2 weeks is estrone sulphate. The plasma concentration of this steroid conjugate is higher than that for estrone or estradiol in postmenopausal women (20) and estrone sulphate can be converted to estrone by estrone sulphatase, an enzyme present in many tissues. As yet there is no information as to the effect of treatment with 4-OHA on circulating concentrations of estrone sulphate. An alternative pathway for estrone formation has been postulated in which dehydroepiandrosterone sulphate is converted to estrone sulphate and thence to estrone via androstenedione enol sulphate (21). However, we were unable to find any evidence to support the presence of such a pathway of estrone formation (22).

Treatment with 4-OHA did not result in any significant change in the MCR androstenedione but for two subjects, a marked increase in the MCR estrone was detected after treatment. For one of the subjects showing an increase in MCR estrone, values for both MCR estrone and MCR androstenedione were lower than we have observed in previous investigations (2, 11). This subject had previously undergone an operation for cholecystectomy and was also taking Aldactone and Lasix for an existing condition. Brodie and Longcope (16) have previously reported that 4-OHA or 4-acetoxyandrostenedione, when administered to rhesus monkeys, produced a marked (18–58%) increase in MCR estrone in four of six animals studied. No increase in MCR estrone occurred for postmenopausal women with breast cancer treated with AG (5).

In order to examine whether, in addition to inhibiting peripheral aromatase activity, 4-OHA also inhibited tumor aromatase activity, this was measured *in vitro* in samples of tumor obtained before and after treatment. While a marked decrease was detected for three of seven tumor samples examined, for two subjects where aromatase activity was initially lower, little change occurred. However, in two other subjects tumor aromatase activity was not inhibited after treatment with 4-OHA. In previous investigations Tilson-Mallet *et al.* (3) and Miller *et al.* (23) have found that the addition of 4-OHA to breast tumor tissue *in vitro* failed to inhibit aromatase activity in some tumors. In contrast to our measurements of *in vitro* tumor aromatase activity before and after treatment with 4-OHA, a parallel study in which *in situ* [3 H]estrone synthesis was measured showed that this was greatly reduced after treatment (24). However, the possibility remains that a proportion of breast tumors may be resistant to the inhibitory effects of 4-OHA. As in the present investigation peripheral conversion of androstenedione to estrone was almost completely inhibited by 4-OHA, it is possible that differences exist between the aromatase enzyme complex present in breast tumors and peripheral tis-

sues. In support of this possibility we have recently failed to inhibit breast tumor aromatase activity with antibodies generated against the placental aromatase complex.⁴ Alternatively concentrations of 4-OHA achieved in some tumors, or parts of tumors, may be insufficient to inhibit aromatase activity.

In addition to measuring tumor aromatase activity *in vitro* we also measured DNA polymerase α activity as a marker of cellular proliferative activity. Although estradiol and not estrone is considered to be the biologically active estrogen, it was reasoned that any reduction in aromatase activity would be associated with a decrease in DNA polymerase α activity, if locally formed estrogen exerts a biological effect. Such a decrease was noted for the majority of tumor samples examined after treatment but it is of interest that for one subject where an increase in tumor aromatase was detected DNA polymerase α activity also increased.

In conclusion, results from this study have shown that treatment with 4-OHA can effectively inhibit the conversion of androstenedione to estrone and that this results in a significant reduction in circulating levels of estrone and estradiol. Aromatase activity, as measured *in vitro* in tumor tissue was not inhibited in all samples examined suggesting that the aromatase enzyme complex in tumor and peripheral tissues may differ or that for some tumors concentrations of 4-OHA high enough to inhibit aromatase activity are not achieved.

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⁴ Unpublished observation.

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