Effect of Aromatase Inhibitors on Growth of Mammary Tumors in a Nude Mouse Model

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ABSTRACT

The effects of aromatase inhibitors 4-hydroxyandrostenedione (4-OHA), CGS 16949A, and CGS 20267, and of the antiestrogen tamoxifen (TAM), were studied on the growth of human breast carcinoma in a nude mouse model. To simulate the postmenopausal breast cancer patient, tumors were formed from inoculates of MCF-7 cells transfected with the human aromatase gene to provide a source of non-ovarian estrogen in ovariectomized mice. Tumor growth was significantly inhibited by all treatments (P < 0.05). Greater reduction in growth occurred in mice treated with TAM combined with aromatase inhibitors than with TAM alone. Tumor progesterone receptor concentrations were unaltered by TAM treatment but were reduced by aromatase inhibitors. Progesterone receptor concentrations correlated with tumor growth. The greatest reduction occurred in tumors of CGS 20267-treated mice in which no progesterone receptors were detected. In the ovariectomized mice used in these studies, uterine weight was maintained by estrogen produced from the tumor. Uterine weight was reduced by aromatase inhibitors but not by TAM treatment. However, there was a significant increase in uterine weight in mice treated with the combination of TAM and 4-OHA. Thus, the agonist effect of TAM was evident when estrogen synthesis was inhibited. The results indicate that aromatase inhibitors have potent effects on mammary tumor growth but lack the estrogenic effects on the uterus observed with TAM. There appeared to be no significant benefit in combining TAM with 4-OHA treatment alone.

INTRODUCTION

The antiestrogen TAM³ has been shown to be significantly more beneficial than chemotherapy for postmenopausal patients with hormone-dependent breast cancer (1). TAM competes with estrogen for the ER and thereby blocks its actions in the tumor. Recently, the selective aromatase inhibitor, 4-OHA, which blocks the production of estrogens, was demonstrated to cause significant tumor regression in breast cancer patients with advanced disease who had relapsed from previous hormonal therapy, usually TAM (2-4). Other new inhibitors are currently in clinical trials. It is now important to determine which of these agents is the most effective against tumor growth and/or whether a combination of agents that act by different mechanisms may be more effective than either one alone. To address these questions, we have recently developed a model for postmenopausal breast cancer suitable for studying these types of compounds (5). In postmenopausal women, estrogens are mainly produced by aromatization of androgens in peripheral or non-ovarian tissue (6). In addition, a number of reports have demonstrated the presence of the aromatase enzyme in some, but not all, breast tumors (7-10). Recently, using reverse transcription-PCR to detect aromatase mRNA in breast tumors, we found that 12 of 15 primary breast tumors investigated contained aromatase mRNA (11).

In the model we described, we have used MCF-7 human breast carcinoma cells transfected with the human aromatase gene (MCF-7Ca; Ref. 12) to provide the non-ovarian source of estrogen. When androstenedione injections (0.1 mg/mouse/day) were administered s.c. to OVX mice, there was a significant and consistent increase in the growth of tumors from MCF-7Ca cells, whereas the growth of tumors from MCF-7 cells transfected with the vector was not affected. The growth rate of the MCF-7Ca tumors in OVX mice supplemented with aromatizable substrate was similar to or greater than that in intact mice.

In this report, we have studied the effects of aromatase inhibitors, 4-OHA (13), an imidazole compound CGS 16949A (14), and a triazole compound CGS 20267 (15) alone and in combination with the antiestrogen TAM on the growth of the MCF-7Ca tumors, on tumor aromatase activity, and on the concentrations of ERs and PRs in tumor and uterine tissue from the nude mice.

MATERIALS AND METHODS

Reagents and Chemicals. [1'H]Androstenedione (24.2 Ci/mmol) and the ER and PR assay kits were purchased from NEN Dupont (Boston, MA). Androstenedione, 4-glucose-6-phosphate, NADPH, glucose-6-phosphate dehydrogenase, and TAM were obtained from Sigma Chemical Co. (St. Louis, MO). 4-Hydroxyandrostenedione was prepared as previously in our laboratory (13). CGS 16949A and CGS 20267 were kindly provided by Dr. Ajay Bhattacharya (Ciba-Geigy Pharmaceutical Co., Basel, Switzerland). A 0.3% solution of hydroxypropyl cellulose, average M, 1,000,000 (Aldrich Chemical Company, Milwaukee, WI) in saline, was autoclaved and used to suspend the injected compounds aromatase, 4-OHA, CGS 16949A, CGS 20267, and TAM.

Athymic Mice. Female BALB/c athymic mice 4-6 weeks of age were purchased from Charles River Breeding Labs (Boston, MA). The animals were housed in a pathogen-free environment under controlled conditions of light and humidity and received food and water ad libitum. Ovariectomy was carried out under fluorohane anesthesia 1-3 days before cell inoculation.

Cell Culture and Inoculation to Athymic Mice. MCF-7 cells stably transfected with the human placental aromatase gene (MCF-7Ca) were generously provided by Dr. Shiang Chen (City of Hope, Duarte, CA; Ref. 12) and were cultured as described previously (4). Subconfluent MCF-7Ca cells resuspended in Matrigel (10 mg/ml) were kindly provided by Dr. Hynda Kleinman, NIH, Bethesda, MD (16, 17). Each mouse received s.c. injections at two sites in each flank with 0.1 ml of the cell suspension (1.75 x 10⁶ cells/site). Growth rates were monitored weekly by measuring the tumors with calipers, and volumes were calculated (13).

Treatments. Injections of 0.1 mg/mouse/day androstenedione were begun on the day following inoculation. Treatments began 10-12 days after androstenedione injections and lasted for 7 weeks. Doses were selected to produce a suboptimal inhibition of tumor growth and were based on those used previously (5). The mice received s.c. injections of 4-OHA (0.5 mg/mouse/day), CGS 16949A (0.25, 0.125, and 0.06 mg/mouse/day), CGS 20267 (0.25 and 0.06 mg/mouse/day), or with the antiestrogen TAM (3 μg/mouse/day). The same doses were used in the animals treated with both TAM and an aromatase inhibitor. Control animals received s.c. injections of vehicle (0.3% hydroxy propyl cellulose, 0.1 ml/mouse/day) daily.

Measurement of Tumor Aromatase Activity. The mice were autopsied 2 h after the last injection, and tumors were removed and stored at −70°C until analyzed. Tumors were homogenized in phosphate buffer with a Polytron
homogenizer at 4°C. Aromatase activity in the tumor homogenates was measured as described previously (5, 18). In brief, 0.9 ml homogenate was incubated with 1 μCi of [1H]androstenedione (4.3 pm) and 0.1 ml of a NADPH generating system (NADP 6.7 mm, glucose 6-phosphate 70 mm, and 25 IU glucose 6-phosphate dehydrogenase) for 2 h at 37°C under an atmosphere of oxygen. Two ml of chloroform were then added to extract the steroids. The aqueous phase was then removed and treated with 2.5% charcoal suspension to eliminate residual steroids. The tritium released during aromatization of [1H]androstenedione to form estrogens plus H2O was measured in a liquid scintillation counter. Aromatase activity was calculated from the amount of H2O produced and expressed as fmol estrogen produced per milligram protein per hour. The protein concentration of the homogenate was measured by Lowry’s method (19).

**Measurement of ERs and PRs.** Measurement of ER and PR levels in tumor and uterine tissues was performed by using radioligand binding assays. Steroid receptor concentrations were determined using RIANEN 125I-labeled estrogen and/or progesterin receptor commercial assay kits (DuPont, Boston, MA). All procedures were performed following kit instructions without modifications using homogenates of frozen tumors and uteri. Briefly, frozen tumors and uteri were pulverized in liquid nitrogen and then homogenized in ice-cold MTG buffer containing 0.01 M Tris-HCl (pH 7.4), 0.0015 M EDTA, 10% glycerol, 0.1% monothioglycerol, and 0.1% sodium azide using a Polytron homogenizer. The tissue:buffer ratio was 100 mg/3 ml. Homogenates were centrifuged at 4,200 rpm for 15 min at 4°C, and then the supernatants were removed and centrifuged at 50,000 × g for 1.5 h at 4°C. Aliquots of supernatants (0.1 ml) were incubated overnight at 4°C with either six different concentrations (1.5–0.04 nmol) of 16α-125I-labeled 3,17β-estradiol (500 Ci/mmol) or with six different concentrations (3.2–0.11 nmol) of (Z)-17α-2-125I-labeled 19-nortestosterone (220 Ci/mmol). Nonspecific binding was determined by addition to the reaction mixture of 100-fold excess of unlabeled diethylstilbestrol or 19-nortestosterone. Unbound steroids were separated and removed by incubation with dextran-coated charcoal for 15 min at 4°C, followed by centrifugation at 4200 rpm for 20 min. The quantity of the receptor-labeled steroid complex was measured by counting in a gamma counter (Clinigamma LKB, Turku, Finland). ER and PR concentrations were analyzed by Scatchard’s method (20) and are expressed as fmol/mg protein. Protein concentrations were determined by the method of Lowry et al. (19).

**Statistics.** The effects of treatment were compared by ANOVA using Scheffe’s F-test. Linear regression analysis was used to determine the correlation between progesterone receptor concentrations and tumor weights.

### RESULTS

Fig. 1 shows the percentage change in total tumor volume in each group. Continuous growth occurred in tumors of the control group of mice following inoculation of the MCF-7Ca cells and daily injections of androstenedione. In comparison, tumor growth was reduced in all of the treated animals. After 42 days of treatment, the reduction in tumor growth was significant (P < 0.05) as determined from the weight of the tumors collected at autopsy (Table 1). Although tumor growth rates were decreased by TAM treatment, further reduction in growth occurred when animals were treated with a combination of TAM and an aromatase inhibitor, 4 OHA or CGS 16949A. Tumor weights of mice treated with CGS 16949A and TAM were significantly less than those of animals treated with TAM alone (P < 0.01). Interestingly, the combination of TAM and 4-OHA reduced the weight of tumors more than treatment with TAM alone, but the effect was less than with 4-OHA alone. Aromatase activity measured 2 h after the last injection was inhibited in all tumors from aromatase inhibitor treated animals (P < 0.01). No inhibition of aromatase activity was observed in tumors of animals treated with TAM only (Fig. 2).

There was no significant effect of aromatase inhibitor treatment on estrogen receptor concentrations in tumors relative to the control level, although TAM tended to decrease and 4-OHA to increase ER concentrations (Fig. 3). The difference in concentrations between these two treatments was significant (P < 0.05). PR concentrations were significantly decreased in the tumors of all mice treated with aromatase inhibitors alone (P < 0.05). A greater reduction was ob-

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**Table 1 Effect of aromatase inhibitors and TAM on tumor and uterine weight**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose</th>
<th>Tumor weight (mg ± SE)</th>
<th>Uterine weight (mg ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3 μg</td>
<td>337 ± 22.5</td>
<td>62.3 ± 4.6</td>
</tr>
<tr>
<td>TAM</td>
<td>0.5 mg</td>
<td>230.5 ± 20.3</td>
<td>60.5 ± 3.2</td>
</tr>
<tr>
<td>4-OHA</td>
<td>3 μg</td>
<td>159.1 ± 20.1</td>
<td>44.9 ± 1.5</td>
</tr>
<tr>
<td>TAM + 4-OHA</td>
<td>3 μg</td>
<td>183.5 ± 17.3</td>
<td>82.8 ± 4.7</td>
</tr>
<tr>
<td>CGS 16949A</td>
<td>0.25 mg</td>
<td>119.0 ± 13.9</td>
<td>15.9 ± 1.7</td>
</tr>
<tr>
<td>TAM + CGS</td>
<td>3 μg</td>
<td>103.4 ± 9.4</td>
<td>61.5 ± 4.7</td>
</tr>
<tr>
<td>Control</td>
<td>4.06 mg</td>
<td>145.8 ± 21.9</td>
<td>42.5 ± 9.0</td>
</tr>
<tr>
<td>CGS 16949A</td>
<td>0.125 mg</td>
<td>88.8 ± 18.8</td>
<td>21.6 ± 3.4</td>
</tr>
<tr>
<td>CGS 2627</td>
<td>0.06 mg</td>
<td>98.2 ± 13.2</td>
<td>20.4 ± 1.4</td>
</tr>
<tr>
<td>CGS 2627</td>
<td>0.25 mg</td>
<td>21.7 ± 3.6</td>
<td>17.8 ± 1.7</td>
</tr>
</tbody>
</table>

* P < 0.01 versus control.

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**Fig. 2. Effect of combined treatment of aromatase inhibitor and antiestrogen on aromatase activity in MCF-7Ca tumors.** Data are from the same experiment as shown in Fig. 1. Tumors were removed, and aromatase activity was measured as described in "Materials and Methods." TAM, tamoxifen; CGS, CGS 16949A; *, P < 0.05; **, P < 0.01 versus control.
served with the CGS compounds, which was significant with the higher dose of CGS 16949A, and reached the limit of detection in mice treated with CGS 20267 (Table 2). There was no significant effect of TAM treatment on PR concentrations in the tumors. When treatments were combined, the PR concentrations were significantly reduced to levels similar to those in animals treated with the aromatase inhibitors only.

The effect of the treatments on the mouse uterus was also studied. The wet weight of the uterus was significantly decreased by 4-OHA, CGS 16949A, and CGS 20267 treatment ($P < 0.05$; Table 1). TAM either alone or in combination with CGS 16949A had no significant effect on the weight of the uterus. However, there was a significant increase in the concentration of ERs in the uterus than in the tumors with aromatase inhibitor treatment, the effect being greater with the CGS compounds (Fig. 4; Table 2). Estrogen receptor levels tended to decrease in all TAM-treated mice relative to the controls. The concentrations of progesterone receptors were decreased by treatment with all doses of aromatase inhibitors alone, and the effect was significant with CGS 16949 at 0.25 mg. However, only one determination was possible with the lower doses of CGS 16949 and CGS 20267 shown in Table 2. TAM also caused a significant decrease in uterine PR to about the same extent as 4-OHA, although the levels of PR were significantly higher in the mice treated with the combination of TAM and 4-OHA than in either group receiving a single agent.

**DISCUSSION**

The results suggest that TAM and the aromatase inhibitors are all effective in preventing the growth of mammary tumors in this model. Inhibition of aromatase activity in the tumors is consistent with the mechanism of action of 4-OHA and the CGS compounds and suggests that estrogen production is reduced in the inhibitor-treated mice. The level of plasma estrogen in nude mice, even in the control animals, is very low, and it was not possible to obtain reliable measurements with current techniques.

The reported doses of TAM that inhibit growth of tumors from MCF-7 cells in nude mice vary over a large range (21–23). Osborne et al. (21) found that 5 μg/day of TAM effectively inhibited tumor growth in nude mice supplemented with estrogen, but that maximal inhibition (40% of control) occurred at the dose of 25 μg/day (21). We found that 5 μg/day was effective in intact mice with tumors of wild-type MCF-7 cells (17). To observe a possible synergistic or additive effect when combined with aromatase inhibitors, 3 μg/day TAM was chosen. Although this is less than the minimal dose used in Osborne’s study, this dose of TAM significantly inhibited tumor growth in OVX mice with MCF-7Ca tumors. However, when mice were treated with the combination of TAM and an aromatase inhibitor, there was a greater reduction in tumor growth and tumor weight than with TAM alone. Tumor weights were similar to those of mice treated with inhibitor alone. The tumor weights of TAM plus 4-OHA-treated mice were slightly but not significantly heavier than those of 4-OHA-treated mice. Thus, there appears to be no additional benefit from combining TAM with this aromatase inhibitor, as inhibitor treatment alone tended to produce greater inhibition of tumor growth at the dose used.

Although a number of proteins are induced as a result of estrogen action in most responsive tissues, induction of the PR is associated with the normal function of estrogen. Therefore, we measured ER and PR concentrations to determine the mechanism of the effects of the treatment. TAM tended to reduce ER concentrations in the tumors but did not reduce PR concentrations. When TAM treatment was combined with that of aromatase inhibitors, tumor PR concentrations were decreased to the same extent as with the inhibitor treatment alone. This suggests that the dose of TAM used in these experiments may not

### Table 2 Effect of nonsteroidal aromatase inhibitors on steroid receptors in mammary tumors and uteri of nude mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ER (fmol/mg pr)</th>
<th>PR (fmol/mg pr)</th>
<th>ER (fmol/mg pr)</th>
<th>PR (fmol/mg pr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>24.4 ± 4.6</td>
<td>62.4 ± 16.6</td>
<td>191</td>
<td>390.4</td>
</tr>
<tr>
<td>CGS 16949</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.06 mg</td>
<td>57.2 ± 15.6</td>
<td>21.1 ± 1.1</td>
<td>315</td>
<td>43.6</td>
</tr>
<tr>
<td>0.125 mg</td>
<td>67.5 ± 6.0</td>
<td>19.5 ± 6.0</td>
<td>388.9</td>
<td>58.3</td>
</tr>
<tr>
<td>CGS 20267</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.06 mg</td>
<td>40.9 ± 10.0</td>
<td>ND</td>
<td>375</td>
<td>75</td>
</tr>
<tr>
<td>0.25 mg</td>
<td>74.5 ± 20.8</td>
<td>ND</td>
<td>496</td>
<td>60</td>
</tr>
</tbody>
</table>

* Tissue from the same mice as in Table 1. ER and PR concentrations, fmol/mg protein. ER values are mean ± SE of three determinations. ND, none detected. 
* Single determinations only. Animals received s.c. injections daily with suspensions of aromatase inhibitors or vehicle (control) for 70 days. Tissue was removed at autopsy for receptor assays. 
* $P < 0.05$. 

![Graph](https://example.com/graph1.png)

**Fig. 3.** Effects of aromatase inhibitors and TAM on ER and PR levels in MCF-7Ca tumors grown in OVX nude mice. ERs and PRs were measured in frozen tumor tissues from the mice shown in Fig. 1 as described in "Materials and Methods." Bars, mean ± SE of three determinations; TAM, tamoxifen; CGS, CGS 16949A; *, $P < 0.05$ versus control; +, $P < 0.05$ versus TAM.

![Graph](https://example.com/graph2.png)

**Fig. 4.** Effects of aromatase inhibitors and TAM on ER and PR levels in uteri of OVX nude mice with MCF-7Ca tumors. ERs and PRs were measured in frozen uterine tissues from the mice shown in Fig. 1 as described in "Materials and Methods." Bars, mean ± SE. TAM, tamoxifen; CGS, CGS 16949A; *, $P < 0.05$ versus control; +, $P < 0.05$ versus TAM.
have been sufficient to block the action of the high local levels of estrogens from inducing PRs in the tumors. Thus, TAM appears to be acting mainly as an estrogen antagonist on tumor tissue and does not appear to exert significant agonist action.

The aromatase inhibitors reduced estrogen synthesis and presumably estrogen concentrations to a level that resulted in no induction of tumor PR concentrations with CGS 20267 and only a small induction of PRs with CGS 16949A (Table 2) and 4-OHA treatment. This suggests that CGS 20267 may produce maximum suppression in estrogen production. Although both CGS compounds are equipotent as aromatase inhibitors in vitro, it is evident that CGS 20267 is more potent in vivo in the mice. This is probably related to the more favorable pharmacokinetic profile of triazole compounds. The effect of treatment by aromatase inhibitors with or without TAM on the PRs correlated with tumor growth (r = 0.87; Fig. 5). This suggests that tumor growth is regulated by the level of estrogen production in the inhibitor-treated mice. The finding that PR levels were not decreased by treatment with TAM alone, whereas tumor growth was partly inhibited, may indicate other antitumor mechanisms not directly related to its antiestrogen effects, such as angiogenesis, as have been suggested recently (24).

As the uterus is also a target of estrogen action, we examined the properties of these agents on the uterus of the tumor-bearing mice. Recently, concerns have been raised about the agonist effect of TAM on the endometrium because there have been reports of cases of endometrial cancer among breast cancer patients treated with TAM (25). Uterine weights were found to be significantly decreased in aromatase inhibitor-treated mice compared to controls. The uteri of the control animals were not atrophic in these experiments, since the MCF-7Ca tumors produced enough estrogen to maintain uterine weight in OVX mice. In contrast to aromatase inhibitors, TAM did not reduce uterine weights. However, in the combined treatment groups, when estrogen synthesis was blocked by CGS 16949A, the uterine weights and PR levels were similar to those of mice treated with TAM alone, suggesting that TAM was acting as an estrogen on the uterus. In mice treated with aromatase inhibitors, there was a much greater increase in uterine ER concentrations relative to the control level than in the tumors (P < 0.05). A greater decrease in uterine ER levels in TAM-treated animals was observed. However, with inhibitor treatment alone, the decrease in PR levels relative to the controls was quite similar for both tumors and the uterus. In contrast, in mice treated with the combination of aromatase inhibitors and TAM, there was a trend for the uterine PR levels to be increased more than in the TAM alone group, suggesting antagonism between the two types of compounds. Thus, the PR values paralleled the changes in uterine weights, although the increase in the uterine weight of the 4-OHA + TAM group was significantly greater than in the group treated with TAM alone (P < 0.05). Since the ER level was increased above the control level when estrogen synthesis was inhibited, it may be that under these conditions TAM exhibits a greater estrogenic response as indicated by a greater induction of PR. Thus, the level of ER may be a factor in determining the estrogen/antiestrogen response of TAM. The result is consistent with findings in other laboratories that TAM has uterotrophic effects in OVX mice and induces PR expression in the uterus (26, 27).

In our previous studies in the rat model, we have observed that 2 weeks of 4-OHA treatment tended to increase uterine weight in both intact and OVX rats and to reduce ER concentrations (27, 28). This response appeared to be similar to that of an androgen, such as dihydrotestosterone, rather than to an estrogen (18). No effect of 4-OHA was found on transcription of the ER in the rat (28). It is possible that decreased ER concentrations may have resulted from enhanced degradation of the receptor due to its instability in the absence of estrogen ligand. Nevertheless, uterine PR concentrations were decreased in the rats as in the mice.

Endocrine therapy with aromatase inhibitors has been demonstrated to be useful for treatment of breast cancer in postmenopausal patients with advanced disease. Most patients chosen for clinical trials of aromatase inhibitors received TAM as first-line therapy. Therefore, it is difficult to compare treatments by different endocrine therapies. Preclinical studies of these drugs have been conducted in in vitro systems and in animal models that mimic premenopausal breast cancer. It has not been possible previously to compare these agents in models relevant to postmenopausal breast cancer. TAM has been widely used and is an established treatment for postmenopausal patients. 4-OHA has only recently been evaluated for its antitumor effect in women (29, 30), whereas the CGS compounds are currently in clinical trials (31). In the model described here, the MCF-7Ca tumors in OVX mice are dependent on estrogens from a nonovarian source. This model simulates the situation of postmenopausal breast cancer patients and appears suitable for studying aromatase inhibitors and ER antagonists. The results indicate that CGS 20267 is highly effective in inhibiting tumor growth. Unlike TAM, the aromatase inhibitors did not have agonist effects on the uterus. It appears that in combination, this antitumor may antagonize the effects of the aromatase inhibitor and may not provide any further benefit than treatment with 4-OHA alone. However, other doses of TAM and the CGS compounds need to be explored before reaching a definitive conclusion about their efficacy in combination. Such studies are in progress.

REFERENCES


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