Effects of Aromatase Inhibitors, Aminoglutethimide, and 4-Hydroxyandrostenedione on Cyclic Rats and Rats with 7,12-Dimethylbenz(a)anthracene-induced Mammary Tumors

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ABSTRACT

4-Hydroxyandrostenedione (4-OHA) is a more potent and specific inhibitor of aromatase (estrogen synthetase) than aminogluthethimide (AG). The two inhibitors were compared in rats with 7,12-dimethylbenz(a)anthracene-induced, hormone-dependent tumors and in normal cyclic rats treated for 4 and 2 weeks, respectively. Ovarian estradiol levels and aromatase activities were not consistently reduced, and tumors regressed in only two of eight rats treated with AG. In animals treated with 4-OHA or 4-OHA:AG, the total tumor volume, estradiol levels, and aromatase activity decreased by >70%. Ovarian weights and plasma luteinizing hormone (LH) levels were also reduced by 4-OHA but increased by AG. Uterine weights were not altered by AG treatment but were increased by 4-OHA. Similar but more consistent results were obtained with these treatments in normal, cyclic rats. In ovariectomized rats, AG had no effect, whereas 4-OHA decreased LH levels and increased uterine weights. The results suggest that, although AG reduces ovarian estrogen secretion by aromatase inhibition, this may lead to an increase in LH secretion. Increased LH may promote ovarian growth and aromatase synthesis, counteracting the inhibitory action of AG to some extent. 4-OHA which inactivates aromatase may also prevent new enzyme synthesis by directly inhibiting gonadotropins. This would result in more effective reduction in ovarian estrogen production by 4-OHA than AG during long-term treatment.

INTRODUCTION

It is now evident that breast cancer patients whose tumors contain significant concentrations of estrogen receptors are likely to respond to hormonal therapy (12). The development of strategies that block the action of estrogens or limit their production should be useful in treating this type of patient. Our approach is to develop potent and safe compounds which selectively inhibit estrogen production. We have identified a number of steroids (4, 16) which are specific inhibitors of aromatase, and several appear to inactivate the enzyme in an irreversible manner (2, 11). 4-OHA is the most potent of these, and its in vivo inhibition of ovarian aromatase and estrogen production in rats (5) as well as in vivo inhibition of peripheral aromatization in monkeys (6, 19) has been reported previously by us. In the first clinical use of this compound, we reported recently that estradiol levels were reduced, measurable tumors were significantly decreased, and soft tissue and bone metastatic lesions were healed in postmenopausal breast cancer patients (7). AG is also an inhibitor of estrogen synthesis by virtue of its effects on cytochrome P-450, a component of the aromatase complex (6, 19). AG, first introduced clinically as an anticonvulsant, has been shown to be beneficial to postmenopausal (15) but not premenopausal breast cancer patients (14).

In this paper, we compared the effectiveness of 4-OHA and AG in the rat with carcinogen (DMBA)-induced mammary tumors and investigated whether the 2 compounds might act synergistically. Since the DMBA-induced tumors are dependent on ovarian steroids, this model resembles the premenopausal rather than postmenopausal breast cancer patient. Although we have demonstrated inhibition of ovarian estrogen secretion and aromatase activity by AG in acute studies (1), we have now determined the long-term effect of this compound on estrogen and gonadotropin levels as well as on mammary tumor growth.

MATERIALS AND METHODS

4-OHA was prepared as described previously (4). AG was kindly donated by Dr. C. A. Brownley, Ciba Pharmaceutical Co., Summit, NJ. [1,2-3H]Androstenedione was obtained from New England Nuclear Corp. and checked for purity on thin-layer chromatography prior to use. [1-3H]Androstenedione was prepared from the latter by overnight treatment in sodium hydroxide in 8 ml of methanol to remove 3H at C-2. [1-3H]Androstenedione was then extracted with methylene dichloride and purified by thin-layer chromatography on silica gel-coated plates (ether:hexane, 3:1).

In Vitro Assay of Aromatase Activity

Microsomes were prepared as described previously from human term placenta (16) and ovaries of rats pretreated for 12 days with 100 IU of pregnant mares' serum gonadotropin (Sigma) injected on alternate days (4). The microsomes, equivalent to 50 mg (wet weight) of tissue, were incubated with [1,2-3H]androstenedione (200,000 dpm/1 μM) in 2 ml of 0.1 M phosphate buffer containing an oxygen-generating system [NADP (5 mg)/glucose 6-phosphate (3 mg)/1 IU glucose 6-phosphate dehydrogenase] at 37°C under an oxygen atmosphere (5). Various concentrations of inhibitors were added in ethanol to the dry flask together with one drop of propylene glycol. The solvent was evaporated prior to adding the microsomal mixture. After 30-min incubation, chloroform was added to the vials. An aliquot of the aqueous phase was removed and mixed with an equal volume of 2.5% charcoal, and after centrifugation, the radioactivity in the supernatant was measured. The amount of estrogen produced was calculated from the tritium released during aromatization of [1,2-3H]androstenedione and converted to 3H2O (18).

Animal Experiments

Tumor-bearing Rats. All animals were female Sprague-Dawley rats from Charles River Breeding Laboratories, and they were housed under controlled conditions of temperature, humidity, and light (14 h) and fed ad libitum. The rats were gavaged at 50 to 55 days of age with 20 mg of DMBA (Sigma) in 2 ml of sesame oil as described (10). Animals which had been exposed to the carcinogen less than 6 months beforehand...
were selected for experiments when at least one tumor per rat had reached a diameter of 2 cm. Tumors were measured with calipers once a week, and the tumor volume was calculated (\(V = \frac{4}{3}\pi r^3\)) (9). Groups of rats were given injections s.c. twice daily with 4-OHA, AG, or 4-OHA:AG in steroid suspending vehicle (Klucl, 0.3% hydroxypropylcellulose). The daily dose of inhibitor was 50 mg/kg, which was the dose found effective previously for 4-OHA (4). Controls were given injections of the vehicle. All AG-treated animals were given 0.9% NaCl solution (saline) to drink. Vaginal smears were taken daily to determine stages of the estrous cycle. On the last day of the experiment, animals were given injections at 9:00 a.m. Three h later, blood (1 ml) was collected from the ovarian vein. Control and AG-treated rats were bled on diestrus, since animals treated with 4-OHA were consistently diestrus. The ovaries and uterus were then removed and weighed. The ovaries were frozen until assayed.

Statistics

Data were analyzed by one-way analysis of variance, followed by Newman-Keuls multiple-range analyses. As indicated in the tables, analyses were performed on log_{10}-transformed data if Cochran's test of the raw data revealed heterogeneous variances.

RESULTS

In Chart 1 is shown the dose response data for inhibition of aromatization of 1 \(\mu\)M androstenedione by 4-OHA and AG. It is interesting to note that the activity of 4-OHA is about 5-fold greater in microsomes from human placenta than from rat ovaries, whereas AG has similar potency in both tissues. The 50%-inhibitory concentrations of the compounds are shown in Table 1. 4-OHA is approximately 60 times more potent than AG in human placental microsomes.

Chart 2 shows the percentage of change in total tumor volume with 4-OHA, AG, or 4-OHA:AG versus controls. In the control group, 2 of 12 tumors regressed, but there were 14 new tumors after 4 weeks, resulting in an increase in total tumor volume. 4-OHA causes marked tumor regression as reported previously (5). After 4 weeks, although one tumor did not regress, the total tumor volume of 18 tumors on 10 rats had decreased by 75%. Four new tumors occurred during the first week of treatment, but all regressed completely by the fourth week. Treatment with AG decreased the volume of 10 of 12 tumors, but at the end of the experiment, there were 11 new tumors, so that there was no decrease in the total tumor volume. The combined treatment of 6 rats with 4-OHA and AG caused tumor regression to a similar extent as with 4-OHA alone. Five of the 6 original tumors...
were less than half their original size after 4 weeks. Although 4 new tumors developed, 3 had regressed completely by the end of the treatment period.

Rats treated with AG alone continued to cycle, whereas 4-OHA-treated animals showed only diestrous smears. All animals were therefore autopsied on diestrous for blood collections. Mean estradiol concentrations in ovarian vein samples and ovarian aromatase activity were decreased in AG-treated rats (Table 2), but the difference was not significant compared to controls. There was considerable variability among the AG-treated rats, but in 4 of 6 rats, values were similar to the controls with only 2 animals having significantly decreased estradiol concentration. However, there was no correlation between the response of tumors and estrogen concentrations. There was a consistent and significant decrease in estradiol secretion and aromatase activity with 4-OHA and also with 4-OHA:AG treatment. LH levels measured in peripheral plasma were significantly reduced by 4-OHA and by 4-OHA:AG in comparison with control values in samples collected from rats in diestrous (Table 2). In contrast, LH values were increased approximately 2-fold by AG in comparison to controls. Prolactin concentrations were very variable, and there was no significant difference between treated and control groups (data not shown). The mean ovarian weight was also increased by AG but decreased by 4-OHA and 4-OHA:AG. Uterine weight was unaffected by AG. Despite reduced estrogen levels, uterine weight was not decreased by 4-OHA or by 4-OHA:AG treatment.

Because of the variability in the concentrations of estradiol during AG treatment and because the cycle pattern, in the control animals as well as the AG-treated rats, was irregular, healthy cycling rats were treated with AG and 4-OHA for 2 weeks. As shown in Table 3, the results were similar to those after 4 weeks of treatment in the tumor-bearing rats. Although there was much less variation in the estradiol values, there was no significant reduction by AG treatment compared to control values. Uterine weights were unaffected by AG. They were also not decreased by 4-OHA. As in the experiment with tumor-bearing rats shown in Table 2, LH values were decreased by 4-OHA and increased (3-fold) by AG relative to the controls.

To determine the mechanism of the effects of these compounds on LH secretion and uterine weight, rats ovariectomized 2 weeks previously were treated for 2 weeks. In these animals, there was no significant difference in the LH values or uterine weights between controls and AG-treated rats (Table 4). However, LH values were markedly suppressed, and uterine weights were increased more than 2-fold by 4-OHA.

**DISCUSSION**

We have reported previously that 4-OHA is a potent inhibitor of aromatase and causes time-dependent inactivation of the enzyme (2, 4). These observations have been confirmed by others (8), and the evidence suggests that 4-OHA may be a kcat or active site-directed inhibitor. AG appears to be a competitive inhibitor of cytochrome P-450 and is a weaker inhibitor of aromatase than 4-OHA. It is not known whether the differences in activity of the 4-OHA in the rat ovarian microsomes versus human placental microsomes are due to species differences or to tissue difference. If the former were true, it implies that 4-OHA may be more active in the human in vivo. In contrast, no difference was noted in the activity of AG in the 2 systems.

In the tumor-bearing rats, there was considerable variability in the response of tumors, estradiol secretion, and aromatase activity to AG treatment. Estradiol secretion was inhibited in 2 rats but not in other animals. Although many of the original tumors decreased in volume, 11 new tumors appeared and increased during treatment, but the overall effect of AG on the tumors was not significantly different from controls (Chart 2). In one animal, there was a marked decrease in both tumor volume and estradiol secretion (<25 pg/ml, value not included in the

### Table 2

**Effect of 4-OHA and AG treatment on rats with DMBA-induced mammary tumor**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ovarian wt (mg)</th>
<th>Aromatase (% of activity/mg of protein)</th>
<th>Estradiol (pg/ml)</th>
<th>LH (ng/ml)</th>
<th>Uterine wt (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>79.6 ± 5.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.10 ± 1.05&lt;sup&gt;e&lt;/sup&gt; (6)</td>
<td>474.0 ± 100.3&lt;sup&gt;e&lt;/sup&gt; (5)</td>
<td>167.7 ± 29.8&lt;sup&gt;e&lt;/sup&gt; (7)</td>
<td>432.3 ± 34.1&lt;sup&gt;e&lt;/sup&gt; (7)</td>
</tr>
<tr>
<td>4-OHA</td>
<td>45.8 ± 5.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.43 ± 0.06&lt;sup&gt;c&lt;/sup&gt; (8)</td>
<td>140.0 ± 18.7&lt;sup&gt;c&lt;/sup&gt; (6)</td>
<td>14.7 ± 6.4&lt;sup&gt;c&lt;/sup&gt; (10)</td>
<td>544.3 ± 21.0&lt;sup&gt;c&lt;/sup&gt; (10)</td>
</tr>
<tr>
<td>AG</td>
<td>117.3 ± 7.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.58 ± 0.25&lt;sup&gt;c&lt;/sup&gt; (6)</td>
<td>326.8 ± 153.4&lt;sup&gt;c&lt;/sup&gt; (5)</td>
<td>297.3 ± 50.8&lt;sup&gt;c&lt;/sup&gt; (7)</td>
<td>424.5 ± 36.3&lt;sup&gt;c&lt;/sup&gt; (8)</td>
</tr>
<tr>
<td>AG:4-OHA</td>
<td>89.8 ± 4.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.67 ± 0.21&lt;sup&gt;c&lt;/sup&gt; (5)</td>
<td>140.5 ± 24.2&lt;sup&gt;c&lt;/sup&gt; (4)</td>
<td>10.8 ± 3.1&lt;sup&gt;c&lt;/sup&gt; (6)</td>
<td>473.2 ± 14.9&lt;sup&gt;c&lt;/sup&gt; (6)</td>
</tr>
</tbody>
</table>

*<sup>a</sup> Statistical analyses of differences were performed on log<sub>10</sub>-transformed data, since Cochran's test of raw values revealed heterogeneous variances.
|<sup>b</sup> Mean ± SE.
|<sup>c</sup> Significance (P < 0.01).
|<sup>d</sup> Numbers in parentheses, number of animals.
|<sup>e</sup> Significance (P < 0.005).

### Table 3

**Effect of 4-OHA and AG on normal cyclic rats**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LH (ng/ml)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Estradiol (pg/ml)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Uterine wt (mg)</th>
<th>Ovarian wt (mg)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>107.3 ± 19.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>433.3 ± 74.9&lt;sup&gt;c&lt;/sup&gt; (6)</td>
<td>300 ± 12.3&lt;sup&gt;c&lt;/sup&gt; (12)</td>
<td>77.2 ± 1.8&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>4-OHA</td>
<td>47.1 ± 12.3&lt;sup&gt;c&lt;/sup&gt; (5)</td>
<td>154.0 ± 14.5&lt;sup&gt;c&lt;/sup&gt; (5)</td>
<td>419 ± 31.8&lt;sup&gt;c&lt;/sup&gt; (6)</td>
<td>68.4 ± 5.6</td>
</tr>
<tr>
<td>AG</td>
<td>322.8 ± 27.1&lt;sup&gt;c&lt;/sup&gt; (7)</td>
<td>294.0 ± 55.0&lt;sup&gt;c&lt;/sup&gt; (5)</td>
<td>367.7 ± 25.4 (6)</td>
<td>191.9 ± 8.0&lt;sup&gt;c&lt;/sup&gt; (7)</td>
</tr>
</tbody>
</table>

*<sup>a</sup> Statistical analyses of differences were performed on log<sub>10</sub>-transformed data, since Cochran's test of raw values revealed heterogeneous variances.
|<sup>b</sup> Mean ± SE.
|<sup>c</sup> Significantly different (P < 0.05).
|<sup>d</sup> Numbers in parentheses, number of animals.
|<sup>e</sup> Significantly different (P < 0.005).
controls. The total tumor volume for these animals increased given injections s.c. of 4-OHA or AG (50 mg/kg/day) twice daily for 2 weeks. Procedures were the same as for Table 1.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LH (ng/ml)</th>
<th>Uterine wt (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1092.9 ± 65.5</td>
<td>102.7 ± 4.9</td>
</tr>
<tr>
<td>4-OHA</td>
<td>71.3 ± 14.0</td>
<td>269.4 ± 18.2</td>
</tr>
<tr>
<td>AG</td>
<td>1148.4 ± 123.1</td>
<td>110.3 ± 5.8</td>
</tr>
</tbody>
</table>

* Statistically analyses of differences were performed on log-transformed data, since Cochran’s test of raw values revealed heterogeneous variances.

** Significantly different (P < 0.005).

mean value for Table 2), but 5 animals secreted more than 190 pg of estradiol per ml and were all within the range of the controls. The total tumor volume for these animals increased approximately 4-fold. Blood could not be collected from 2 other animals. Estrous cycles also continued during AG treatment. The estradiol secretion by control animals was quite variable, and the estradiol levels were reduced compared to controls, and daily vaginal smears were leukocytic, consistent with low estradiol production.

We have observed previously that 4-OHA and AG decreased ovarian aromatase activity and estrogen secretion to a similar extent in acute experiments in which rats were given injections on the morning of proestrus, and tissues and blood were collected 3 h later (1). However, in long-term experiments of 2 and 4 weeks, it is evident that estradiol suppression is not maintained by AG to the same degree. The initial 90% inhibition of ovarian estradiol synthesis by AG may lead to increased LH levels via feedback regulatory mechanisms in the intact rat. Reflex increases in LH and FSH were observed in ovariectomized patients treated with AG (14). Increased gonadotropins may tend to stimulate aromatase synthesis by the ovaries and counteract the inhibitory effects of AG to some extent. After 2 weeks in the normal cycling animals, there was a 50% reduction in the mean value of estradiol which, due to variation, was not significantly different from the control value. However, after 4 weeks of treatment, estrogen levels in 5 of 6 tumor-bearing animals was within the range of values for control animals. This amount of estradiol was sufficient to maintain the uterine weight comparable to intactcontrol rats. AG appeared to have no direct effect on either the uterus or pituitary in ovariectomized rats, whereas marked reduction in LH levels by 4-OHA suggests a direct action of this compound independent of aromatase inhibition. The effect on LH secretion* as well as on the uterus appears to be due to weak androgenic activity (<1% testosterone) of 4-OHA (20) which may contribute to its efficacy in causing regression of DMBA-induced mammary tumors. Thus, 4-OHA by more potent aromatase inhibition and gonadotropin suppression may prevent new enzyme synthesis and follicular development by the ovary, resulting in a greater and sustained reduction in estradiol production than AG. This activity may be of importance in considering 4-OHA as treatment for patients with functional ovaries. However, in postmenopausal breast cancer patients, inhibition of gonadotropins would not be expected to be advant-

tageous, since peripheral aromatase appears not to be regulated by LH and FSH (13).

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REFERENCES


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