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Report

Effects of the aromatase inhibitor 4-hydroxyandrostenedione and the antiandrogen flutamide on growth and steroid levels in DMBA-induced rat mammary tumors

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Key words: aromatase inhibitor, 4-hydroxyandrostenedione, antiandrogen, flutamide, steroid metabolism, DMBA-induced rat mammary tumors, breast cancer

Summary

Using dimethylbenz(a)anthracene (DMBA)-induced mammary tumors in the rat as model, comparison was made of the effect of treatment for 20 days with the aromatase inhibitor 4-hydroxyandrostenedione (4-OH-A) (7.5 mg, twice daily) or the antiandragen flutamide (5 mg, twice daily) on tumor growth as well as on plasma and tumor content of estrogens, androgens, and their precursors and metabolites. Tumor number and size were markedly decreased following treatment with either drug, the effect of treatment being more important on size than number, and on new tumors which developed during treatment than on tumors already present at start of treatment. Treatment with the aromatase inhibitor 4-OH-A caused a parallel decrease in plasma and tumor levels of pregnenolone (Preg), progesterone (P), and 17-OH P, while there was a marked increase in dehydroepiandrosterone (DHEA), androst-5-ene-3β,17β-diol (Δ^5-diol), androstenedione (Δ^4-dione), testosterone (T), androstane-3α, 17β-diol (3α-diol), and androstane-3β,17β-diol (3β-diol), with no significant change in dihydrotestosterone (DHT) and 17β-estradiol levels. The marked increase in tissue T content coupled to a decrease in P levels could well contribute to the inhibition of tumor growth induced by 4-OH-A. Flutamide, on the other hand, caused a marked fall in plasma and tissue levels of Preg, 17-OH Preg, P, and 17-OH P, with no significant change in the concentration of the other steroids, thus suggesting a possible role of the fall in tissue P levels in the inhibition of tumor growth. Since both drugs are potent inhibitors of DMBA-induced tumor growth in intact animals, better knowledge of their mechanism of action should add to our understanding of the multiple endocrine factors controlling the growth of these tumors.

Introduction

Mammary carcinoma induced in the rat by dimethylbenz(a)anthracene (DMBA) is the most widely used *in vivo* model of human breast cancer [1–3]. In addition to the well known stimulatory effect of estrogens and prolactin [1–4], androgens have been reported to exert variable effects on the growth of this tumor [5, 6]. Both DMBA-induced mammary tumors in the rat [7, 8] and a large proportion of human breast cancer [9–11] possess androgen receptors.

It is now well demonstrated that procedures which reduce estrogen activity or limit its produc-

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tion, on one hand, or which reduce circulating levels of prolactin, on the other hand, cause a reduction in the number and size of these tumors [2, 3, 7, 8, 12–18]. Moreover, tumor growth can be reinitiated in ovariectomized animals by treatment with estrogens, prolactin, or C_{19}–Δ^5 adrenal steroids [4, 8, 19, 20]. Since we have recently found that the antiandrogen flutamide can have a marked inhibitory effect on the growth of DMBA-induced mammary tumors in the rat [21], and it is well established that the aromatase inhibitor 4-hydroxyandrostenedione causes tumor regression in the same hormone-dependent mammary tumor model [16–18], we have compared the effect of these two compounds on the growth of DMBA-induced mammary carcinoma and have measured plasma and tumor content of estrogens, androgens, and their precursors following treatment with both compounds.

Materials and methods

Animals

Mammary tumors were induced in female Sprague-Dawley (Crl : CD(H(SD)Br) rats (obtained from Charles River Canada Inc., St. Constant, Quebec) at 50 to 55 days of age by a single intragastric administration of 20 mg of DMBA (Sigma Chemicals Co., St. Louis, Mo) in 1 ml of corn oil. Animals were housed two per cage under a regimen of 14 h of light (lights on between 05:00 and 19:00 H) and 10 h of darkness in a temperature (22 ± 1° C)-controlled environment. Purina rat chow and water were given ad libitum.

Treatments

Three to four months after DMBA administration, animals with palpable tumors were selected and tumor number and size were recorded. The animals were then divided into three groups: intact controls, intact animals treated with 4-hydroxyandrostenedione (4-OH-A), and intact rats treated with flutamide (FLU). At the start of treatment, each group had similar average and distribution of tumor number and size.

The animals of the appropriate groups were treated twice daily for 20 days with 7.5 mg of 4-OH-A [22] or 5 mg of FLU injected subcutaneously in 0.5 ml of 1% gelatin – 0.9% NaCl. Intact controls received the vehicle alone. The aromatase inhibitor 4-OH-A was synthesized in our laboratory as described previously [22], while the nonsteroidal antiandrogen flutamide (α,α,α-trifluoro-2-methyl-4′-nitro-m-propionotoluidine) was kindly provided by Drs. T.L. Nagabuschan and R. Neri, Schering Corporation, Kenilworth, New Jersey. Both compounds were first dissolved in a small volume of ethanol before further dilution with 1% gelatin – 0.9% NaCl.

Animals were examined for the presence of mammary tumors by palpation and the number of tumors per rat was recorded. Tumor measurements were performed on the day before starting treatment and at 3- to 4-day intervals for 20 days. In addition, the two largest perpendicular diameters of each tumor were measured with calipers and the product of these diameters was used to estimate tumor size as described [3, 8]. At the end of the 20-day treatment period, the animals were killed by decapitation. Tumors were immediately removed, freed from connective and adipose tissue, frozen on dry ice and stored at −80° C until assayed. Plasma samples were stored at −20° C until assayed.

Steroid radioimmunoassays

Steroid concentrations were measured by radioimmunoassays following methanol and diethyl ether extraction for tissue samples and diethyl ether extraction for plasma samples and chromatography on LH-20 columns as described in detail elsewhere [23]. All the samples were chromatographed and radioimmunuassayed (RIA) simultaneously.

Calculations

Radioimmunoassay data were analyzed using a

program based on Model 11 of Rodbard and Lewald [24]. All data are expressed as means ± SEM of duplicate determinations of individual samples. Statistical significance was calculated according to the multiple-range test of Duncan-Kramer [25].

Results

Effects of 4-hydroxyandrostenedione and flutamide on tumor growth

As illustrated in Fig. 1, there is a steady increase in the number of tumors in the intact control animals from 1.81 ± 0.21 to 2.77 ± 0.34 tumors/animal during the 20 days of observation. In the animals treated with 4-hydroxyandrostenedione and flutamide, tumor number decreased to 0.92 ± 0.25 and 1.67 ± 0.30, respectively, thus causing a highly significant ($p<0.01$) reduction in tumor number when compared with intact control animals.

When only those tumors present at the beginning of treatment are considered (Fig. 2), tumor number remained approximately constant in the intact controls from 1.81 ± 0.21 to 1.92 ± 0.21 (N.S.). In the group of animals treated with 4-OH-A and FLU, tumor number decreased from 1.80 ± 0.34 to 0.92 ± 0.26 ($p<0.01$ versus intact controls) and from 1.69 ± 0.34 to 1.47 ± 0.31 (N.S.), respectively. On the other hand, it can be seen in Fig. 3 that, while in the intact control animals the number of new tumors is 0.85 ± 0.22 ($p<0.01$ versus the animals treated with 4-OH-A or FLU), no new tumors could be detected in intact + 4-OH-A-treated animals while 0.20 ± 0.11 tumor was measured per animal treated for 20 days with FLU.

Fig. 4 shows the effect of the same treatments on average tumor area (cm^2/rat). While average tumor

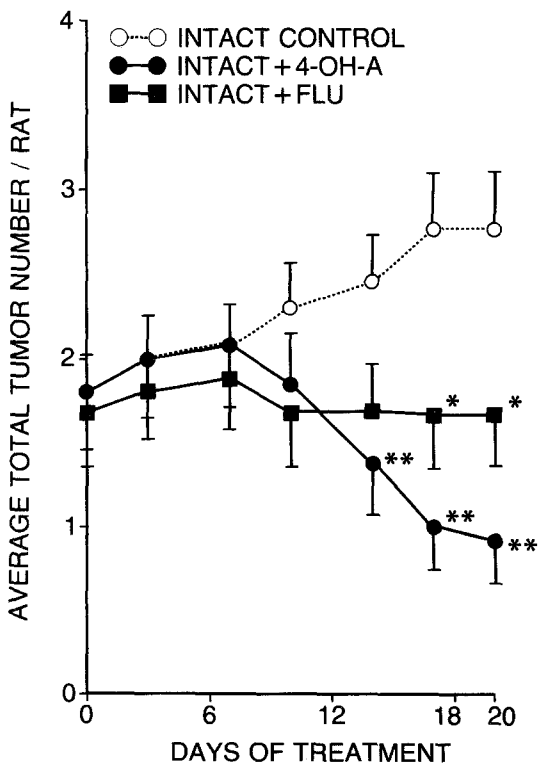

Fig. 1. Effect of 20-day treatment of intact animals with 4-HYDROXYANDROSTENEDIONE (7.5 mg, twice daily) or FLUTAMIDE (5 mg, twice daily) on the total number of DMBA-induced mammary tumors in the rat (* $p<0.05$, ** $p<0.01$).

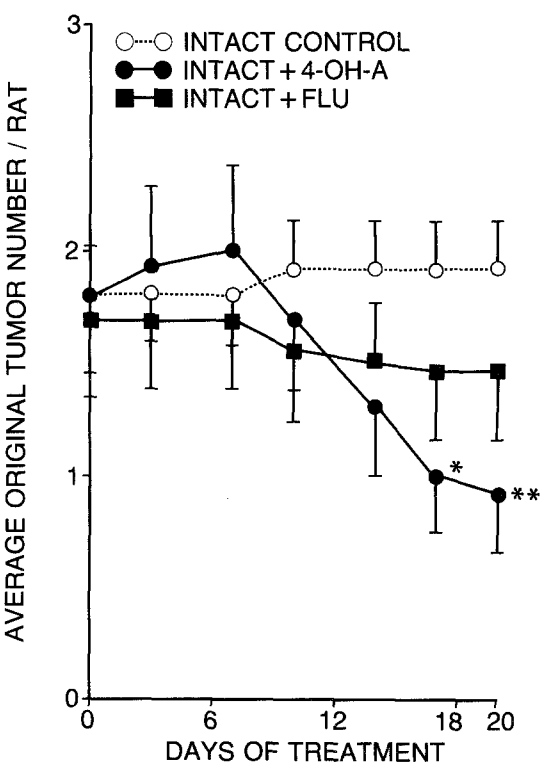

Fig. 2. Effect of 20-day treatment of intact animals with 4-HYDROXYANDROSTENEDIONE (7.5 mg, twice daily) or FLUTAMIDE (5 mg, twice daily) on the number of original (present at the start of treatment) DMBA-induced mammary tumors in the rat (* $p<0.05$, ** $p<0.01$).

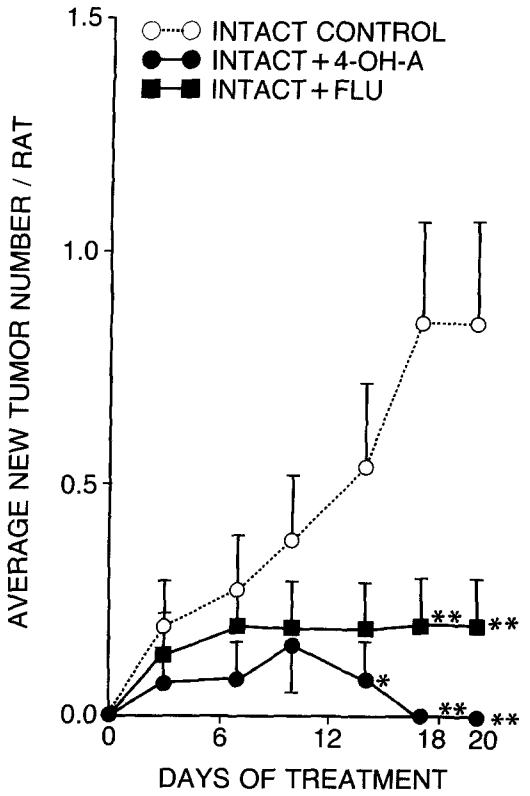

Fig. 3. Effect of 20-day treatment of intact animals with 4-HYDROXYANDROSTENEDIONE (7.5 mg, twice daily) or FLUTAMIDE (5 mg, twice daily) on the number of newly-developed DMBA-induced mammary tumors in the rat (* $p<0.05$, ** $p<0.01$).

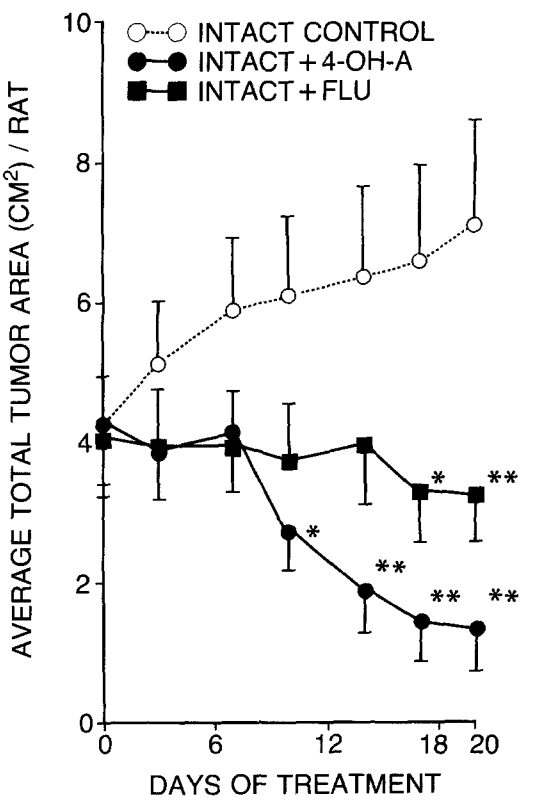

Fig. 4. Effect of 20-day treatment of intact animals with 4-HYDROXYANDROSTENEDIONE (7.5 mg, twice daily) or FLUTAMIDE (5 mg, twice daily) on the average total area (cm^2) of DMBA-induced mammary tumors in the rat (* $p<0.05$, ** $p<0.01$).

area is $7.11 \pm 1.50\,cm^2$ in intact control animals at the end of the experiment, it is dramatically reduced to $1.35 \pm 0.61\,cm^2$ ($p<0.01$) and $3.27 \pm 0.67\,cm^2$ ($p<0.01$) after 20 days of treatment with 4-OH-A or FLU, respectively. When only the original tumors are measured at the end of the 20-day observation period, the average tumor area (cm^2) in the intact + 4-OH-A and intact + FLU groups was also dramatically reduced from a value of 6.30 ± 1.70 to 1.35 ± 0.61 ($p<0.01$) and 3.10 ± 0.70 ($p<0.05$), respectively (Fig. 5).

When only new tumors are considered, the average area in intact control animals reached $0.88 \pm 0.23\,cm^2$ while in animals treated with 4-OH-A or FLU, the average new tumor area was decreased to the lower limit of detection ($p<0.01$) and $0.17 \pm 0.11\,cm^2$ ($p<0.01$), respectively (Fig. 6).

Effects on plasma and tumor tissue steroid levels

While plasma pregnenolone (Preg) levels are 1.4 ± 0.2 ng/ml in intact animals, they are reduced to 0.3 ± 0.06 ng/ml ($p<0.01$) and 0.6 ± 0.1 ng/ml ($p<0.01$) in animals treated with 4-OH-A and FLU, respectively. It can also be seen that tissue levels of Preg are significantly ($p<0.05$) decreased after 20 days of treatment, with values of 1.8 ± 0.3 ng and 2.1 ± 0.5 ng/g tissue in animals treated with 4-OH-A and FLU, respectively, as compared to 3.6 ± 0.7 ng/g tissue in intact animals. The reduction in the plasma levels of 17-OH pregnenolone (17-OH Preg) by treatment with 4-OH-A and FLU was not significant (Fig. 7C), while FLU caused a highly significant ($p<0.01$) decrease in the tissue concentration of 17-OH Preg from 0.8 ± 0.1 to 0.3 ± 0.1 ng/g tissue.

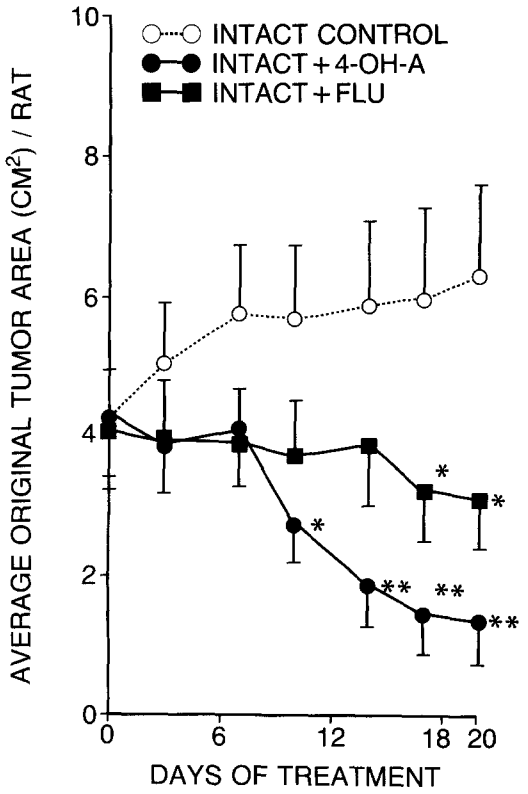

Fig. 5. Effect of 20-day treatment of intact animals with 4-HYDROXYANDROSTENEDIONE (7.5 mg, twice daily) or FLUTAMIDE (5 mg, twice daily) on the area (cm^2) of original (present at the start of treatment) DMBA-induced mammary tumors in the rat (* $p<0.05$, ** $p<0.01$).

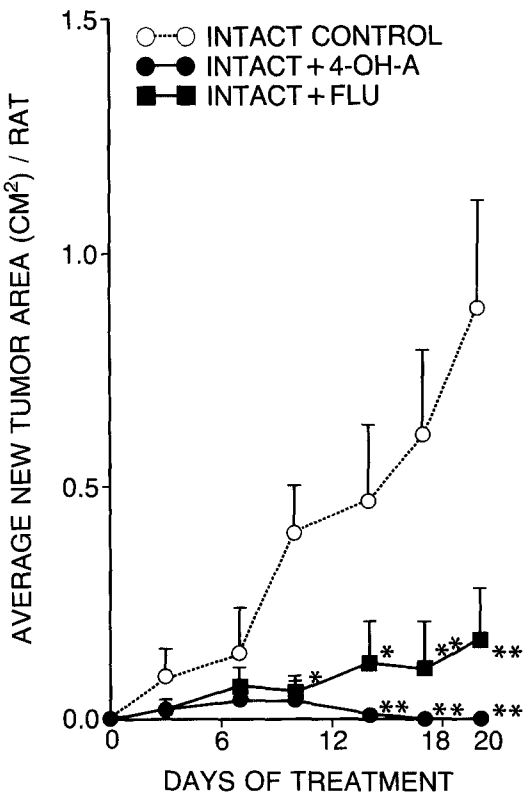

Fig. 6. Effect of 20-day treatment of intact animals with 4-HYDROXYANDROSTENEDIONE (7.5 mg, twice daily) or FLUTAMIDE (5 mg, twice daily) on the area (cm^2) of newly-developed DMBA-induced tumors in the rat (* $p<0.05$, ** $p<0.01$).

As illustrated in Fig. 8A and Fig. 8B, treatment with 4-OH-A or FLU caused a 63 to 81% decrease in the plasma and tissue levels of progesterone (P). In fact, the plasma P values were reduced from 10.9 ± 1.7 ng/ml in intact animals to 2.1 ± 1.4 ng/ml ($p<0.01$) and 4.0 ± 1.4 ng/ml ($p<0.01$) in the plasma of 4-OH-A- and FLU-treated animals, respectively, and from 2.9 ± 0.6 ng/g tissue in intact controls to 0.9 ± 0.1 ng/g ($p<0.01$) and 0.9 ± 0.2 ng/g tissue ($p<0.01$) in 4-OH-A- and FLU-treated animals, respectively. The plasma levels of 17-OH-P were significantly ($p<0.01$) decreased following 4-OH-A and FLU treatment (Fig. 8C), while the reduction in the tissue levels of the steroid was not significant (Fig. 8D).

It can be seen in Figs. 9, 10, 11 and 12 that the plasma levels of dehydroepiandrosterone (DHEA), androst-5-ene-3β,17-diol (Δ^5-diol), androstenedione (Δ^4-dione), testosterone (T), androstane-3α,17β-diol (3α-diol), and androstane-3β,17β-diol (3β-diol) were markedly increased after 20 days of treatment with 4-OH-A, while FLU had no significant effect. Parallel effects were observed on the tissue levels of DHEA, Δ^5-diol, Δ^4-dione, T, and 3β-diol, while no significant change was observed on the tumor content of 3α-diol after 4-OH-A treatment. FLU, on the other hand, had no effect on the tissue content of any of the above-mentioned steroids. As illustrated in Figs. 11 and 12, plasma and tissue levels of DHT and 17β-estradiol were not significantly affected after 20 days of treatment with 4-OH-A or FLU.

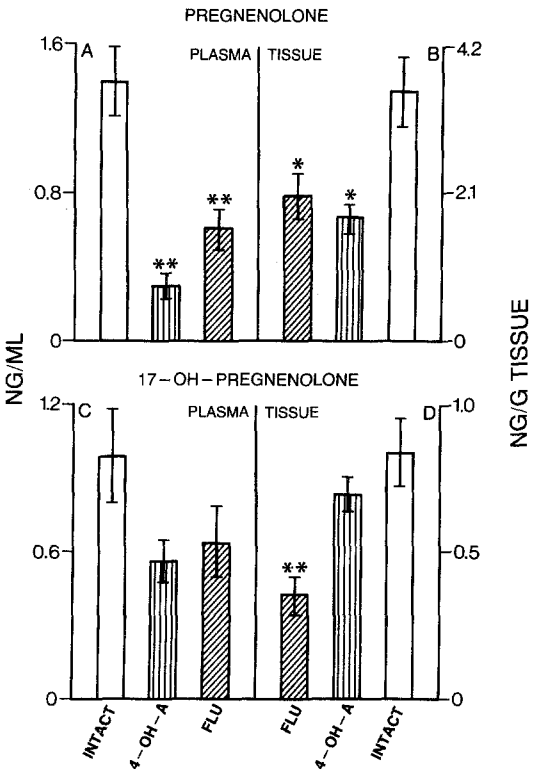

Fig. 7. Effect of 20-day treatment of intact animals with 4-HYDROXYANDROSTENEDIONE (7.5 mg, twice daily) or FLUTAMIDE (5 mg, twice daily) on the plasma concentrations of pregnenolone (A) and 17-OH-pregnenolone (C), and on the intratissular concentrations of pregnenolone (B) and 17-OH-pregnenolone (D).

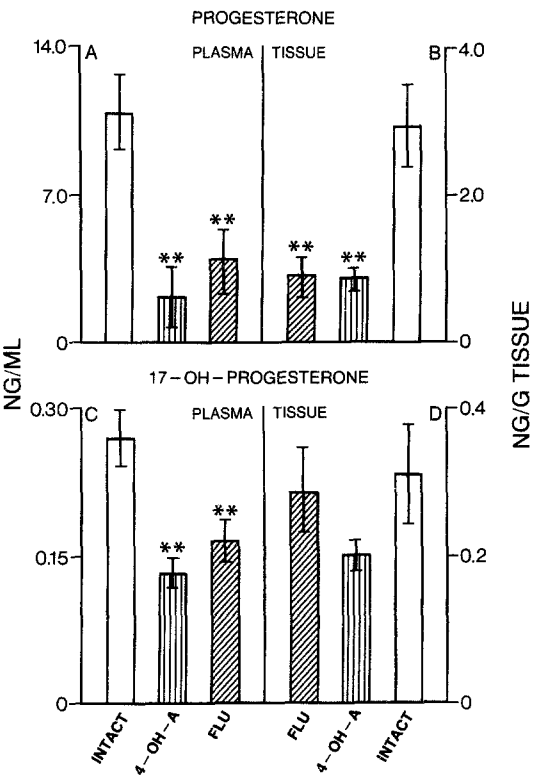

Fig. 8. Effect of 20-day treatment of intact animals with 4-HYDROXYANDROSTENEDIONE (7.5 mg, twice daily) or FLUTAMIDE (5 mg, twice daily) on the plasma concentrations of progesterone (A) and 17-OH-progesterone (C), and on the intratissular concentrations of progesterone (B) and 17-OH-progesterone (D).

Discussion

The present data demonstrate that the aromatase inhibitor 4-hydroxyandrostenedione and the anti-androgen flutamide, at comparable doses, have a marked inhibitory effect on the growth of DMBA-induced mammary tumors in the rat. The most important inhibitory effect of the two drugs is on the number and size of new tumors which developed during the 20-day observation period, while their effect on tumors afready present at the start of treatment is of lower amplitude. While an average of 0.85 ± 0.20 new tumor appeared in each intact control rat during the 20-day period of observation, there were no measurable new tumors in animals treated for 20 days with 4-OH-A while only 0.20 ± 0.11 new tumor per rat was found in the group of animals treated with flutamide. On the other hand, the number of original tumors decreased from 1.92 ± 0.2 to 0.92 ± 0.26 and 1.47 ± 0.31 in animals treated with 4-OH-A or FLU, respectively.

In addition to their strong inhibitory effect on the total number of tumors, both treatments caused a marked inhibition of tumor size which was of greater amplitude than the effect observed on tumor number. The average size of original tumors was reduced by 79% ($p<0.01$) and 51% ($p<0.01$) after 20 days of treatment with 4-OH-A or FLU, respectively, while, when original tumor number was considered, the inhibitory effects of 4-OH-A and FLU were 52% ($P<0.01$) and 23% (N.S.), respectively. The present data indicate that the inhibitory effect of both 4-OH-4 and FLU on tumors present at the start of treatment is of greater amplitude on

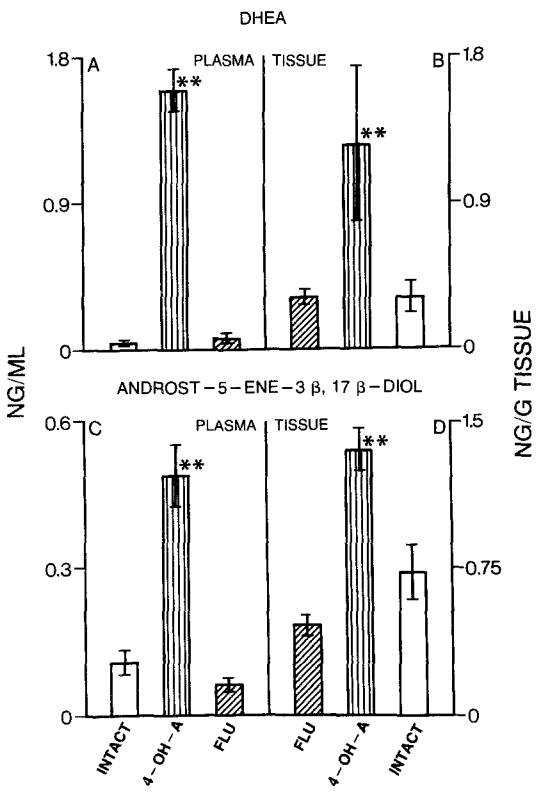

Fig. 9. Effect of 20-day treatment of intact animals with 4-HYDROXYANDROSTENEDIONE (7.5 mg, twice daily) or FLUTAMIDE (5 mg, twice daily) on the plasma concentrations of DHEA (A) and androst-5-ene-3β,17β-diol (C), and on the intratissular concentrations of DHEA (B) and androst-5-ene-3β,17β-diol (D).

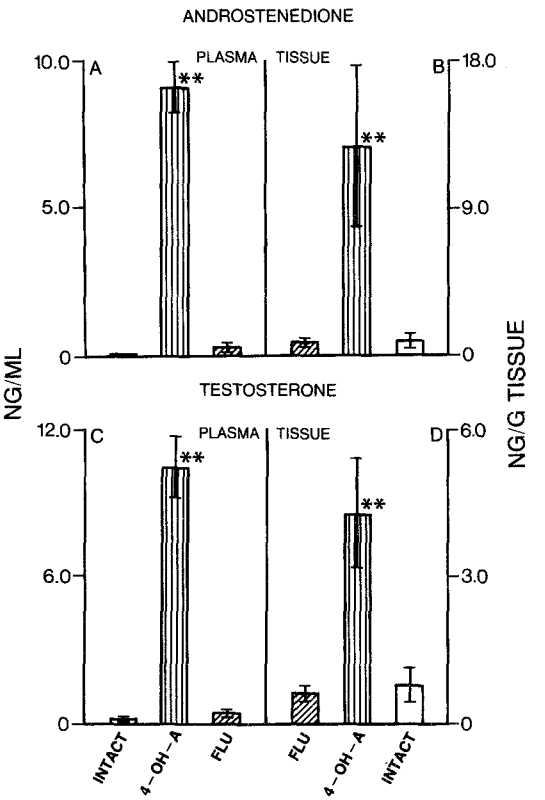

Fig. 10. Effect of 20-day treatment of intact animals with 4-HYDROXYANDROSTENEDIONE (7.5 mg, twice daily) or FLUTAMIDE (5 mg, twice daily) on the plasma concentrations of androstendione (A) and testosterone (C), and on the intratissular concentrations of androstenedione (B) and testosterone (D).

tumor size than on number. However, when new tumors as opposed to original tumors are considered, the marked inhibitory effect of both drugs is comparable on tumor number and size. In fact, no new tumor could be found after 20 days of treatment with 4-OH-A, while both tumor size and number were reduced by 80% after FLU treatment.

While the activity of 4-OH-A as aromatase inhibitor is well known [22, 26, 27] and its inhibitory effect on the growth of DMBA-induced mammary carcinoma has been described [16, 28], the data available on the changes in steroid levels in the plasma were limited to 17β-estradiol and no information had been obtained on the concentration of steroids in tumor tissue. The more unexpected finding may be the marked reduction in plasma and tissue levels of pregnenolone, progesterone, and 17-OH progesterone after 4-OH-A treatment. Considered alone, such data indicate a corresponding decrease in gonadotropin secretion, side-chain cleavage enzymatic activity, or/and an increased 17,20 lyase activity. The high plasma T levels measured during treatment could also play a role in these changes through a feedback inhibitory effect of androgens at the hypothalamo-pituitary level [29, 30] resulting in a decrease in gonadotropin secretion. The intrinsic androgenic activity of 4-OH-A (31) is also likely to exert some direct inhibitory action on gonadotropin secretion. The high plasma and tissue levels of androgens in the presence of decreased concentrations of pregnenolone,

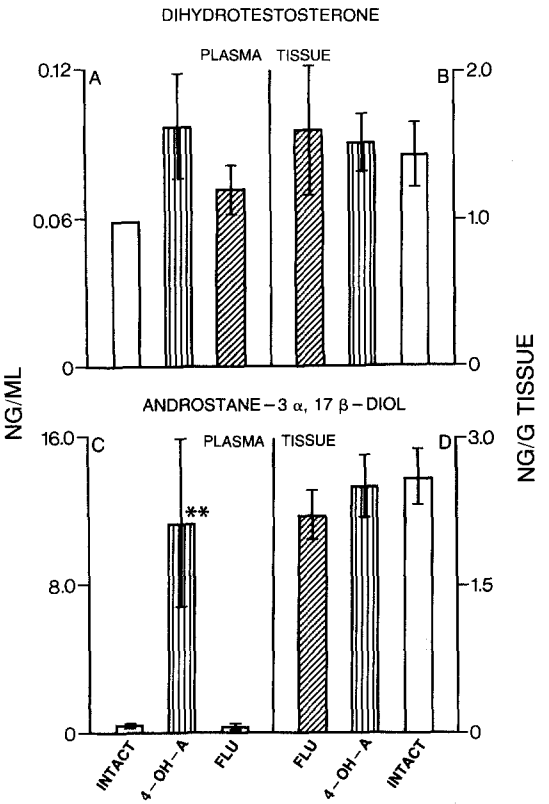

Fig. 11. Effect of 20-day treatment of intact animals with 4-HYDROXYANDROSTENEDIONE (7.5 mg, twice daily) or FLUTAMIDE (5 mg, twice daily) on the plasma concentrations of dihydrotestosterone (A) and androstane-3α,17β-diol (C), and on the intratissular concentrations of dihydrotestosterone (B) and androstane-3α,17β-diol (D).

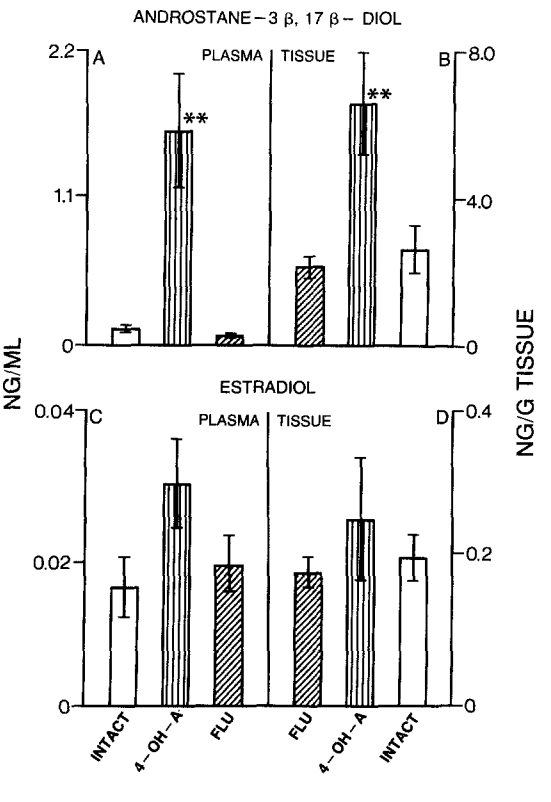

Fig. 12. Effect of 20-day treatment of intact animals with 4-HYDROXYANDROSTENEDIONE (7.5 mg, twice daily) or FLUTAMIDE (5 mg, twice daily) on the plasma concentrations of androstane-3β,17β-diol (A) and estradiol (C) and on the intratissular concentrations of androstane-3β,17β-diol (B) and estradiol (D).

progesterone, and 17-OH progesterone indicate that although gonadotropin secretion was likely decreased by 4-OH-A treatment, sufficient precursor steroids remained for a highly significant inhibitory effect of the drug on aromatase activity which resulted in an accumulation of the more immediate precursors.

It can be seen in Fig. 12 that treatment with the aromatase inhibitor 4-OH-A had no significant effect on either plasma or tissue levels of E_2. Variable levels of plasma E_2 have been reported previously after treatment with 4-OH-A [18]. After 4 weeks of treatment with 4-OH-A at the dose of 50 mg/kg, ovarian E_2 levels were reduced by 70% [18]. Although an efficient blockade of aromatase activity is clearly indicated by the elevated plasma concentrations of T, Δ^4-dione, DHEA, and Δ^5-diol, the absence of effect of 4-OH-A on E_2 levels after 20 days of treatment might well be due to a readjustment at some enzymatic step under 4-OH-A treatment. Since the stage of the estrous cycle was not monitored, it is also possible that an effect of 4-OH-A on the estrous cycle could affect plasma and tissue E_2 levels and explain the lack of difference of estrogen levels between control and 4-OH-A-treated animals.

Since androgens have been reported to inhibit the growth of DMBA-induced mammary tumors [6], the marked increase in the tumor concentration of T (Fig. 10D) coupled with the intrinsic androgenic activity of 4-OH-A [31] might play an important role in the strong inhibitory effect of

4-OH-A treatment. Moreover, since progestins have been reported to stimulate the growth of this model tumor [4, 32], the marked loss in the tumor concentration of progesterone induced by 4-OH-A treatment might also be involved in the inhibitory effect of long-term treatment with the inhibitor 4-OH-A. However, since Δ^5-diol has clearly been shown to be estrogenic and to stimulate the growth of DMBA-induced mammary tumors [20], the high tumor levels of this C_{19}–Δ^5 steroid during 4-OH-A treatment are likely to exert some stimulatory effect on tumor growth, an effect most likely reversed by the high T levels, the androgenic activity of 4-OH-A itself, and the low plasma P levels. With the present knowledge of the multiple changes in the tissue concentrations of steroids having opposite effects on tumor growth, it is clear that the inhibitory effect of 4-OH-A on DMBA-induced tumor growth is complex and includes more than the well known inhibition of aromatase activity.

At the dose used (5 mg, twice daily), the present data show that flutamide is a potent inhibitor of DMBA-induced tumor growth. The detailed study of plasma and tumor content of a large series of steroids potentially involved in control of tumor growth offers one possible explanation for the effect of flutamide, namely the marked fall in plasma and tissue content of progesterone. Since progesterone has been reported to stimulate DMBA-induced mammary carcinoma growth [4, 32], the marked inhibition of growth induced by treatment of intact animals with flutamide could result, to an unknown extent, from the fall in tissue progesterone levels. Although no data are available on the potential effects of pregnenolone and 17-OH-pregnenolone on DMBA-induced tumor growth, the marked fall in plasma and tissue levels of pregnenolone and 17-OH pregnenolone could also play a role through unknown mechanisms. The decrease in plasma pregnenolone and progesterone suggests a decrease in cholesterol side-chain cleavage enzymatic activity which seems unrelated to the presently known actions of flutamide. In fact, this compound is known as a pure non-steroidal antiandrogen having no androgenic, estrogenic, progestational, glucocorticoid, or other hormonal or antihormonal activity [33–36].

Although 4-hydroxyandrostenedione and flutamide act through different mechanisms, the present data show that both are potent inhibitors of growth of the estrogen-sensitive DMBA-induced mammary carcinoma in the rat. The magnitude of the effects observed suggests further investigation of the mechanisms of action of these two drugs in order to gain better knowledge of the endocrine control of breast cancer growth.

Acknowledgements

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