Antagonism of Development and Growth of 7,12-Dimethylbenz(a)anthracene-induced Rat Mammary Tumors by the Antiestrogen U 23,469 and Effects on Estrogen and Progesterone Receptors

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SUMMARY

This study analyzes the effectiveness of a nonsteroidal antiestrogen, cis-(3-[p-(1,2,3,4-tetrahydro-6-methoxy-2-phenyl-1-naphthyl)phenoxy]-1,2-propanediol (U 23,469) previously shown to be potent in antagonizing estrogen-induced uterine growth, in preventing the development of 7,12-dimethylbenz(a)anthracene (DMBA)-induced rat mammary tumors and in eliciting the regression of established tumors; the study also attempts to elucidate the mechanisms of the tumor antagonism of U 23,469. Virgin female Sprague-Dawley rats that receive DMBA at 47 to 50 days of age and then receive U 23,469 (250 μg s.c. in 0.15 M NaCl daily) have a greatly reduced number of mammary tumors and a markedly decreased tumor area. Treatment with U 23,469 for increasing time periods (3, 6, or 12 weeks) beginning 2 weeks after DMBA results in a progressive decrease in tumor size and number and a progressive delay in onset of tumor appearance. U 23,469 treatment beginning 1 week after DMBA or given prior to DMBA is even more effective. The time course of tumor regression (3 months after DMBA) by U 23,469 or ovariectomy is similar, with 50% regression in ca. 2 weeks, and both elicit regression of almost all tumors (>90%).

After ovariectomy, cytosol progesterone receptor levels are greatly diminished in tumors and uteri, while cytosol estrogen receptor (ER) levels are high; in both tissues little (ca. one-third) of ER is in the nucleus. During U 23,469 treatment, cytosol ER content is very low in regressing tumor and uterus and over 90% of ER is in the nucleus; cytosol progesterone receptor is slightly depressed in the uterus but is at the untreated level in mammary tumor. These receptor studies suggest that the effectiveness of this antiestrogen in antagonizing mammary tumor development and growth may reside in its ability markedly to perturb the distribution of ER, maintaining over 90% of ER in the nucleus with concomitant low levels of cytoplasmic ER, a situation that may render the mammary tissue insensitive to the animal's own endogenous estrogens.

INTRODUCTION

Antiestrogens are a class of compounds that prevent estrogens from expressing their full effects on estrogen target tissues; as such, they antagonize a variety of estrogen-dependent processes (5, 19, 25, 31). Theoretically then, these compounds hold the potential of being noninvasive, nonsurgical agents capable of controlling the growth of estrogen-dependent tumors; in fact, studies in the DMBA-induced rat mammary tumor system (6, 13, 15, 23, 30) and clinical trials with humans with breast cancer (2, 8, 12) indicate that antiestrogens are effective in controlling the growth of hormone-dependent mammary tumors. However, the mechanisms by which antiestrogens act remain largely unknown.

In the studies reported here, we analyze the effectiveness of a nonsteroidal antiestrogen previously shown to be potent in antagonizing estrogen-induced uterine growth (10), in preventing the development of DMBA-induced rat mammary tumors, and in eliciting the regression of established tumors; and we attempt to elucidate the mechanisms of its tumor antagonism. Inasmuch as one of the problems frequently associated with antiestrogen therapy has been the development of photosensitivity in patients (2, 8), we have utilized in this study an antiestrogen, U 23,469 (26), which is structurally related to the antiestrogen nafoxidine (see Chart 1) but which is nonphototoxic and hence may prove to be more suitable for human use (see "Discussion").

This report indicates that this antiestrogen is an effective antagonist of mammary tumor development and growth, that it is most effective when given near the time of carcinogen administration, and that if administered after carcinogen it must be given for a long period of time to be maximally effective. Receptor studies suggest that the effectiveness of this antiestrogen may reside in its ability to maintain
most of the estrogen receptor in the nucleus with concomi-
tant low levels of cytoplasmic estrogen receptor, a situation
that may render the mammary tissue and uterus insensitive
to the animal’s own endogenous estrogens.

MATERIALS AND METHODS

Animals. Virgin female Sprague-Dawley rats (from The
Holtzman Company, Madison, Wis.) were housed in a light
(14 hr/day)- and temperature (72°F)-controlled room and
given a diet of Purina laboratory chow (Ralston Purina Co.,
St. Louis, Mo.) and tap water ad libitum. Rats, at 47 to 50
days of age, received a single i.v. injection (1 ml) of a lipid
emulsion containing 5 mg of DMBA. Beginning at 1 month
after the DMBA treatment, all animals were palpated once a
week for the appearance of mammary tumors. The number
of tumors per rat and body weight of the rats were recorded.
The tumor size was measured in 2 dimensions (length x
width) with calipers. When the majority of the tumors in a
given rat reached a minimum of 0.6 cm in each diameter,
the rats were ovariectomized or received antiestrogen injec-
tions (250 µg U 23,469 in 0.5 ml of 0.15 M NaCl containing
5% ethanol s.c. daily). In some experiments, after tumor
regression was followed, estradiol (5 µg in 0.5 ml 0.15 M
NaCl s.c. daily) was administered to the animals to see the
possible regrowth of the regressed tumors. Concomitant
changes in mammary tumor and uterine estrogen receptor
levels, receptor distribution between nucleus and cyto-
plasm, and the level of progesterone receptor in cytoplasm
were determined.

Chemicals and Reagents. [1,2-3H(N)]Progesterone (55.7
Ci/mmmole), [1,2,6,7-3H(N)]progesterone (103.7 Cl/mmmole),
[6,7-3H(N)]estradiol (47.9 Ci/mmmole), and [2,4,6,7-
3H(N)]estradiol (91.3 Cl/mmmole) were obtained from New
England Nuclear, Boston, Mass., and were checked for
radiochemical purity by thin-layer chromatography. The
radiochemical purity of [1,2-3H(N)]progesterone (55.7
Ci/mmmole) and [6,7-3H(N)]estradiol (47.9 Ci/mmmole) were
found in mammary tumor tissue and uterus at 30° and 45
min; hence these conditions were used in all assays. Mam-
mary tumor values were equally good under exchange con-
ditions of 15° for 4 hr. Following the period of exchange,
the tubes were chilled in ice for 10 min, and a volume of hydrox-
ylapatite slurry equal to that of the nuclear pellet was added.
(We have found that the addition of this small amount of
hydroxylapatite prevents loss of receptor during the subse-
quent washes.) The pellet then was washed 3 times with 3
ml of 50 mM Tris, pH 7.3, at 0°, each wash followed by
centrifugation at 800 x g for 10 min, and the bound 17β-
estradiol was determined by ethanol extraction of the
pellet (room temperature, 30 min). The cytosol binding as-
say was performed on the 180,000 x g supernatant fraction
as described by Katzenellenbogen et al. (22).

Cytosol Binding of [3H]Progesterone. Cytosol binding of
[3H]progesterone was performed according to the method
of Feil et al. (9) with modification. The tissues were homog-
ized in iced 10 mM Tris:1.5 mM EDTA:12 mM thiglycerol:20% glycerol buffer, pH 7.4, at 25° (ca. 100 mg tissue per ml buffer) with motor-driven Kontes all-glass tissue
grinders. The homogenates were spun at 180,000 x g for 60
min at 4° in a Beckman L2-65B ultracentrifuge (type 65
rotor, 46,000 rpm) to yield the high-speed supernatant (cy-
itosol). The cytosols were pretreated with 10⁻⁸ M cortisol at 0°
for 30 min to exclude corticosteroid-binding globulin and
glucocorticoid receptor binding and were then incubated
with 3 x 10⁻⁸ M [3H]progesterone or with [3H]progesterone
plus 100 x radioinert progesterone for 3 hr at 0° to deter-
mine total and nonspecific binding sites, respectively.
Pre-treatment with 10⁻⁷ M cortisol gave identical results. After
the 3-hr equilibration period, aliquots were assayed by add-
ing charcoal:dextran slurry (slurry:cytosol, 1:4) to adsorb
unbound steroid for 2 and 5 min for uterus and tumor
sample, respectively. The supernatant was obtained by
spinning at 800 x g for 15 min and was counted in Tri-
ton:xylene scintillation fluid (0.3% PPO, 0.02% POPOP, and
25% Triton X-114 in xylene) at 40% efficiency. We have

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body weight i.p.; Pitman-Moore, Inc., Washington Cross-
ing, N. J.). The mammary tumors and uterus were quickly
excised and weighed, and weighed portions (about one-
third of the tissue) were put in iced 10 mM Tris:1.5 mM
EDTA:12 mM thiglycerol:20% glycerol buffer, pH 7.4, at
25°, or in iced Tris:EDTA:sodium azide buffer (about two-
thirds of the tissue). All the procedures are performed at 0°
unless otherwise stated. Tissues were homogenized with
Kontes all-glass tissue grinders at approximately 100 mg
tissue per ml buffer (w/v). The nuclear binding sites were
determined as described previously by Katzenellenbogen
(18) with slight modifications. The 800 x g for 20 min
nuclear pellet was washed 3 times with 3 ml iced TE buffer
and was then resuspended in TE buffer. Each tube contain-
ing nuclear pellet from 10 mg of original tissue in 0.9 ml TE
buffer was incubated at 30° for 45 min with 3 x 10⁻⁸ M 17β-
estradiol to determine total binding or with 17β-
estradiol plus 100 x radioinert diethylstilbestrol to deter-
mine the nonspecific binding. These incubation conditions
were found to be optimal for both uterine and mammary
tumor tissues. Nuclear exchange assays with tumor or ma-
ture uterine tissue were run at 15°, 25°, 30°, and 37° for 20
and 45 min and for 2, 4, and 21 hr. Optimal exchange was
found in mammary tumor tissue and uterus at 30° and 45
min; hence these conditions were used in all assays. Mam-
mary tumor values were equally good under exchange con-
ditions of 15° for 4 hr. Following the period of exchange,
the tubes were chilled in ice for 10 min, and a volume of hydrox-
ylapatite slurry equal to that of the nuclear pellet was added.
(We have found that the addition of this small amount of
hydroxylapatite prevents loss of receptor during the subse-
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estradiol was determined by ethanol extraction of the
pellet (room temperature, 30 min). The cytosol binding as-
say was performed on the 180,000 x g supernatant fraction
as described by Katzenellenbogen et al. (22).

Determination of Nuclear and Cytosol Estrogen-binding
Sites. Rats were killed by rapid decapitation. In some exper-
iments, where indicated, for standardization of the endo-
ctrine status of the animals, they were ovariectomized 19 hr
before sacrifice. Bilateral ovariectomy was performed by
the dorsal route under Innovar anesthesia (0.028 ml/100 g
characterized the progesterone receptors determined in this manner to be specific for progestins with high affinity binding of progesterone, $K_d$ of $10^{-8}$ M, determined by Scatchard analysis. We have found values for progesterone receptor levels to be similar in the cases in which $[^3H]$-17,21-dimethyl-19-norpregna-4,9-diene-3,20-dione binding as determined above.

**Protein and DNA Assays.** Protein concentration of cytosol samples was assayed by the method of Lowry et al. (27), with bovine serum albumin as standard, and DNA concentration of nuclear preparations was determined by the Burton assay (3, 20).

**Statistics.** The significance of differences between treatment groups was examined by Student's $t$ test.

**RESULTS**

Chart 2 (left middle) shows the experimental protocol that we have utilized to determine the effectiveness of the antiestrogen U 23,469 in preventing mammary tumor development and growth. This antiestrogen, U 23,469, is structurally related to the nonsteroidal antiestrogen, U 11,100A, also known as nafoxidine, but it differs from nafoxidine in possessing a different side chain and a different central ring (Chart 1). Female Sprague-Dawley rats received DMBA at 47 to 50 days of age, and then following a 2-week period of rats were ovariectomized or they received U 23,469 injections of 250 $\mu$g/day either for 1 day or for 3, 6, or 12 weeks. Another group was pretreated with U 23,469 for 10 days before DMBA administration, and another group received 15 weeks of U 23,469 exposure starting at only 1 week after DMBA.

This chart (Chart 2) shows the average tumor area per rat of animals in these different treatment groups. In animals receiving only DMBA, palpable tumors began to appear about 40 days after DMBA treatment, and they grew rapidly so that the average tumor area per rat was some 4 to 5 sq cm by 140 days. Animals that were ovariectomized at 2 weeks after DMBA showed no development of tumors during the assay period (160 days). Even antiestrogen treatment for only 1 day greatly reduced tumor size. Treatment with U 23,469 for 10 days before DMBA administration and another group received 15 weeks of U 23,469 exposure starting at only 1 week after DMBA.

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Table 1 summarizes data on the influence of these treatments on tumor development. This table indicates the average number of tumors per rat, the tumor incidence, and the average tumor area per rat for the different treatment groups at 90 and at 140 days after DMBA.

In animals receiving only DMBA (Table 1, Line 1), the average number of tumors per rat is about 2 at 90 days and increases to ca. 3 at 140 days. Seventy-nine % of treated rats have at least 1 tumor at 90 days and tumor incidence increases to 90% by 140 days. After ovariectomy, no tumors appeared during the assay period. After 1 day or 3, 6, or 12 weeks of U 23,469 treatment or after U 23,469 pretreatment, the average number of tumors per rat (Table 1, Column 1) is greatly reduced as is tumor area per rat (Table 1, Column 3) at both 90 and 140 days, yet tumor incidence is high; by 140 days, well over 50% of animals have at least 1 tumor, although the tumors are tiny. In contrast, treatment with U 23,469 for 15 weeks, where the treatment is started after only 1 week of DMBA exposure, appears completely to eliminate tumor development, as does ovariectomy.

Chart 3 indicates that the effect of antiestrogen on inhibition of mammary tumor development and growth is probably not due to a debilitating effect on health of the rats as monitored by rate of weight gain. The rate of weight gain is slightly slower in animals during antiestrogen treatment than in controls (DMBA only), and the rate increases after antiestrogen treatment is stopped. Terenius (30) has reported a similar, depressive effect of the antiestrogen MER-25 on body weight gain in rats. As expected (7), weight gain...
Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>90 days after DMBA</th>
<th>140 days after DMBA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean no. of tumors/rat</td>
<td>Tumor incidence (rats/tumor/total rats)</td>
</tr>
<tr>
<td>DMBA only</td>
<td>1.80 (0-8)*</td>
<td>46/58 (79)*</td>
</tr>
<tr>
<td>Ovariectomy</td>
<td>0 (0)</td>
<td>0/13 (0)</td>
</tr>
<tr>
<td>1-day U 23,469</td>
<td>1.20 (0-4)</td>
<td>7/9 (78)</td>
</tr>
<tr>
<td>3-wk U 23,469</td>
<td>1.10 (0-4)</td>
<td>4/9 (44)</td>
</tr>
<tr>
<td>6-wk U 23,469</td>
<td>0.55 (0-2)</td>
<td>4/9 (44)</td>
</tr>
<tr>
<td>12-wk U 23,469</td>
<td>0.44 (0-1)</td>
<td>4/9 (44)</td>
</tr>
<tr>
<td>10-day U 23,469 pretreatment</td>
<td>0.22 (0-1)</td>
<td>2/10 (20)</td>
</tr>
<tr>
<td>15-wk U 23,469</td>
<td>0 (0)</td>
<td>0/8 (0)</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, range.

† Numbers in parentheses, percentage of rats with tumors.

Chart 3. Influence of antiestrogen treatments or of ovariectomy on animal body weights. Rats (10 to 13/group) received DMBA at 47 to 50 days of age and at 2 weeks after DMBA rats were ovariectomized (OVX) or received U 23,469 (U-23) injections of 250 μg/day for 3, 6, or 12 weeks. Another group (10 day pretreat) received U23,469 injections for 10 days prior to the administration of DMBA and no further treatments thereafter. Control rats (58 rats) are those receiving DMBA only. Values represent the mean weight for each group of rats.

is significantly greater (ca. 50 g more over the assay period) in ovariectomized rats than in controls. Hence, there appears to be no correlation between tumor incidence or growth and body weight of the rats.

Chart 4 shows the effects of U 23,469 treatment or ovariectomy on the regression of established tumors. Here, 15 animals have been used per group. It is seen that ovariectomy or administration of U 23,469 elicits regression of almost all tumors, and they both show a similar time course with 50% regression elicited in approximately 2 weeks.

As seen in Chart 5, estradiol (5 μg/rat/day) is able to reactivate these regressed tumors. Tumors caused to regress by ovariectomy or by U 23,469 treatment begin growth soon after the administration of estradiol.

The effects of these different treatments on estrogen and progesterone receptor levels in mammary tumors are summarized in Table 2. We have determined here the levels of nuclear-estrogen receptor, cytosol-estrogen receptor, and cytosol-progesterone receptor and the ratio of nuclear to total estrogen receptor. In tumors induced by DMBA, we find approximately 0.2 pmole of cytoplasmic estrogen receptor per 100 mg tissue [59 ± 9 (n = 30) fmole/mg cytosol protein], and approximately one-half of the receptor is in the nucleus. Likewise, high levels of progesterone cytosol receptor are found. This appears to be the case whether tumors are assayed from cycling animals at random stages during the estrous cycle (Table 2, Group 1a) or whether tumors are assayed from animals at 19 hr after ovariectomy (Table 2, Group 1b). Hence, these data have been pooled (Group 1c) and are used for statistical comparison with the other experimental treatment groups (Table 2, Groups 2 to 5).

The statistically significant changes induced by the treatments are as follows. After U 23,469 treatment (Table 2, Group 4), as tumors regress, approximately 90% of the receptor is found in the nucleus. After ovariectomy (Table 2, Group 2), little (30%) of the total receptor is in the nucleus.
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and cytoplasmic receptor levels are high; as expected in the absence of ovarian estrogen, the progesterone cytosol receptor level is very low. In tumors regressing under U 23,469 treatment (Table 2, Group 4), the progesterone receptor level is considerably higher than that seen in mammary tumors regressing due to ovariectomy. Receptor levels were also studied during tumor reactivation by estradiol. In both cases (Table 2, Groups 3 and 5), receptor assays were done 24 hr after the last injection of estradiol, and it is seen that estradiol treatment shifts the estrogen receptor distribution to that more resembling the DMBA-only group (Table 2, Group 1c).

Chart 5. The ability of estradiol (E2) to elicit regrowth of tumors caused to regress by U 23,469 (U-23) treatment or ovariectomy (OVX). Animals received DMBA at 47 days of age, and at the times indicated (arrows) rats (6/group) were ovariectomized or received U 23,469 injections (250 µg s.c. in 0.15 M NaCl per day) for 28 days, prior to administration of estradiol (5 µg s.c. in 0.15 M NaCl per day) for 27 days. Values represent the average tumor area per rat for the 6 rats in each group.

Table 2
Mammary tumor estrogen and progesterone receptor levels

<table>
<thead>
<tr>
<th>Treatment</th>
<th>E · Rn</th>
<th>E · Rn + E · Rc</th>
<th>pmoles/100 mg tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a. DMBA only (no 19 hr OVX)</td>
<td>0.28 ± 0.09 (20)</td>
<td>0.19 ± 0.03 (19)</td>
<td>1.33 ± 0.18 (10)</td>
</tr>
<tr>
<td>1b. DMBA only* (19 hr OVX)</td>
<td>0.19 ± 0.03 (22)</td>
<td>0.25 ± 0.06 (11)</td>
<td>1.48 ± 0.15 (20)</td>
</tr>
<tr>
<td>1c. DMBA only (pooled)</td>
<td>0.23 ± 0.04 (42)</td>
<td>0.21 ± 0.03 (30)</td>
<td>1.43 ± 0.12 (30)</td>
</tr>
<tr>
<td>2. OVX-regressing*</td>
<td>0.33 ± 0.07 (8)</td>
<td>0.79 ± 0.31 (6)</td>
<td>1.04 ± 0.07 (6)</td>
</tr>
<tr>
<td>3. OVX → E2*</td>
<td>0.26 ± 0.06 (5)</td>
<td>0.16 ± 0.04 (5)</td>
<td>0.42 ± 0.16* (5)</td>
</tr>
<tr>
<td>4. U 23,469-regressing*</td>
<td>0.54 ± 0.06* (18)</td>
<td>0.06 ± 0.02* (18)</td>
<td>1.14 ± 0.23 (17)</td>
</tr>
<tr>
<td>5. U 23,469 → E2*</td>
<td>0.21 ± 0.03 (10)</td>
<td>0.15 ± 0.05 (7)</td>
<td>1.05 ± 0.06 (11)</td>
</tr>
</tbody>
</table>

* E · Rn, nuclear-estrogen receptor; E · Rc, cytosol-estrogen receptor; P · Rc, cytosol-progesterone receptor; OVX, ovariectomy; E2, 17β-estradiol.

= Mean ± S.E.
* Numbers in parentheses, number of tumors assayed.
* Regressing tumors were assayed 3 to 5 weeks after OVX.
* p < 0.01 versus DMBA-only pooled value.
* At 3 weeks after OVX, each rat received s.c. injections of 5 µg E2 in 0.5 ml 0.15 M NaCl daily 6 days/week for 3 weeks. The tumors were assayed 24 hr after the last E2 injection.
* Each rat received s.c. injections of 250 µg U 23,469 in 0.5 ml 0.15 M NaCl daily 6 days/week for approximately 4 weeks. The tumors were assayed 24 hr after the last U 23,469 injection and 19 hr after bilateral OVX.
* p < 0.05 versus DMBA-only pooled value.
* After 4 weeks of U 23,469 injections, each rat received E2 injections for 4 weeks. Tumors were assayed 24 hr after the last E2 injection.

Table 3
Uterine estrogen and progesterone receptor levels

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>Uterine wt (mg)</th>
<th>E · Rn</th>
<th>E · Rn + E · Rc</th>
<th>pmoles/100 mg tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a. DMBA only (no 19-hr OVX)</td>
<td>422 ± 31 (8)</td>
<td>0.40 ± 0.07 (12)</td>
<td>0.66 ± 0.19 (12)</td>
<td>1.69 ± 0.28 (8)</td>
</tr>
<tr>
<td>1b. DMBA only (19-hr OVX)</td>
<td>433 ± 31 (7)</td>
<td>0.38 ± 0.12 (7)</td>
<td>0.62 ± 0.22 (6)</td>
<td>1.58 ± 0.25 (6)</td>
</tr>
<tr>
<td>1c. DMBA only (pooled)</td>
<td>428 ± 21 (15)</td>
<td>0.39 ± 0.06 (19)</td>
<td>0.65 ± 0.14 (18)</td>
<td>1.64 ± 0.19 (14)</td>
</tr>
<tr>
<td>2. OVX-regressing</td>
<td>156 ± 19 (10)</td>
<td>0.69 ± 0.10 (12)</td>
<td>0.93 ± 0.20 (12)</td>
<td>0.22 ± 0.04* (10)</td>
</tr>
<tr>
<td>3. OVX → E2*</td>
<td>422 ± 25 (4)</td>
<td>0.30 ± 0.04 (4)</td>
<td>1.36 ± 0.22* (4)</td>
<td>2.04 ± 0.11 (3)</td>
</tr>
<tr>
<td>4. U 23,469-regressing</td>
<td>281 ± 19 (12)</td>
<td>0.90 ± 0.09* (17)</td>
<td>0.06 ± 0.02* (17)</td>
<td>0.98 ± 0.17* (15)</td>
</tr>
<tr>
<td>5. U 23,469 → E2*</td>
<td>481 ± 37 (7)</td>
<td>0.32 ± 0.03 (9)</td>
<td>0.53 ± 0.14 (9)</td>
<td>1.07 ± 0.20* (9)</td>
</tr>
</tbody>
</table>

* Treatments are the same as those indicated in Table 2.
* For abbreviations, see Table 2, Footnote a.
* Mean ± S.E.
* Numbers in parentheses, number of uteri assayed.
* p < 0.01 versus DMBA-only pooled value.
* p < 0.05 versus DMBA-only pooled value.
* p < 0.1 versus DMBA-only pooled value.
In uteri of such animals a generally similar picture is seen (Table 3). After U 23,469 treatment (Table 3, Group 4), most of the estrogen receptor is found in the nucleus, 94%, whereas in ovariectomized animals (Table 3, Group 2), most of the uterine estrogen receptor is cytoplasmic (43% is nuclear). After ovariectomy the cytosol progesterone receptor level is very low, whereas progesterone receptor levels are high but are significantly below the DMBA-only group (Table 3, Group 5) animals increases uterine weight to that of the ovariectomized animals, although uteri of antiestrogen-treated rats are also far below the control weight. This may reflect some uterotrophic, estrogenic activity of the U 23,469 compound (10). Administration of estradiol for 4 weeks to ovariectomized (Table 3, Group 3) or U 23,469-treated (Table 3, Group 5) animals increases uterine weight to that of the control (Table 3, Group 1c) and also increases the level of progesterone receptor especially markedly in the ovariectomized animals. Estradiol also strongly reverses the distribution of the estrogen receptor after U 23,469 prior treatment, returning it to that resembling the DMBA-only control group (less than one-half is nuclear).

**DISCUSSION**

The ability of an antiestrogen to arrest the growth of already established tumors and to elicit tumor regression is no doubt the most important parameter in terms of potential significance in therapy of human breast cancer. Clinical trials with the antiestrogen nafoxidine (2, 8) indicate tumor significance in therapy of human breast cancer. Clinical ton level is very low, whereas progesterone receptor levels whereas in ovaniectomized animals (Table 3, Group 2), most of the estrogen receptor is found in the nucleus, 94%, whereas in ovaniectomized animals (Table 3, Group 2), most of the uterine estrogen receptor is cytoplasmic (43% is nuclear). After ovariectomy the cytosol progesterone receptor level is very low, whereas progesterone receptor levels are high but are significantly below the DMBA-only group (Table 3, Group 5) animals increases uterine weight to that of the control (Table 3, Group 1c) and also increases the level of progesterone receptor especially markedly in the ovariectomized animals. Estradiol also strongly reverses the distribution of the estrogen receptor after U 23,469 prior treatment, returning it to that resembling the DMBA-only control group (less than one-half is nuclear).

Pretreatment with antiestrogen for 10 days prior to ad-

administration of carcinogen results in a very prolonged lag period before any tumors appear, and tumor size and number are greatly reduced. Treatment with antiestrogen for 10 days beginning immediately after DMBA is even slightly more effective than is pretreatment (T-L. S. Tsai and B. S. Katzenellenbogen, data not presented). Such treatment near the time of DMBA administration, which apparently renders the mammary tissue refractory to the carcinogenic effect of DMBA, is as effective as is giving antiestrogen for 12 weeks if antiestrogen treatment is delayed for 2 weeks after exposure to DMBA. Only 1 day of antiestrogen treat-

ment at 2 weeks after DMBA also results in a considerable diminution in tumor size, although tumor incidence is high and there is no delay in the onset of tumor appearance.

Although several antiestrogens such as nafoxidine (13), RU-16117 (23), CI-628 (6), MER-25 (30), and tamoxifen (15) have been shown to inhibit mammary tumor growth and, in the cases studied, to elicit regression of the majority of mammary tumors induced by DMBA (13, 23), the mecha-

nisms by which these compounds act as mammary tumor antagonists are not known. Conceivably, the antagonistic action of an antiestrogen could take place at any of the stages of estrogen interaction with the receptor mechanism of target cells or at hypothetical control points postrecep-

tor; furthermore, they might also influence the growth of DMBA-induced mammary tumors by affecting the levels of prolactin or prolactin receptor.

Many recent studies have focused on elucidating the mechanisms by which antiestrogens inhibit estradiol-in-

duced growth of the uterus. These studies indicate that the level of cytoplasmic receptor is frequently correlated with the extent of responsiveness to estrogenic hormones (1, 19) and that the antagonistic action of antiestrogens appears to derive from their ability to effect a marked perturbation in the subcellular distribution of receptor, whereby very little of receptor (ca. 10%) is cytoplasmic and further estrogen receptor accumulation (most likely synthesis) is blocked (4, 5, 10, 19). This appears to be the case in both uterine and mammary tumor tissue in the adult animals that we have studied here. Jordan and Dowse (16) have also recently reported that animals treated with the antiestrogen tamoxifen *in vivo* have low levels of cytosol estrogen receptor in uterus and mammary tumor. Other studies (17, 29) have likewise suggested the initial action of these compounds to be at the tissue level, since no difference was observed in the levels of plasma prolactin and plasma estradiol between animals with regressed tumors and those in which tumors continued to grow. Likewise, plasma prolactin levels are highly variable in patients on antiestrogen therapy (11).

The role of the progesterone receptor in mammary tumor regression in response to endocrine ablative or additive therapy is presently unknown. Since the progesterone receptor is an estrogen-induced protein, it has been sug-

gested that the presence of the progesterone receptor in a tumor sample prior to treatment may be a useful indicator of its responsiveness to endocrine therapy (14). However, it is apparent from the studies reported here that the levels of tumor cytoplasmic progesterone receptor during endocrine intervention are not necessarily correlated with tumor regression or growth antagonism as in antiestrogen-in-
duced regression, progesterone receptor levels are only slightly reduced from the control while in ovariectomy-induced regression, progesterone receptor levels are extremely low (Table 2).

There is recent evidence that high doses of estrogen decrease the magnitude of prolactin binding in mammary tumors and retard mammary tumor growth (24). Antiestrogens might also influence mammary tumor growth through such a mechanism. However, it is possible that the antagonism of mammary tumor development and growth by antiestrogens may be accounted for completely by their ability to maintain most of the estrogen receptor in the nucleus with concomitant low levels of cytoplasmic estrogen receptor, a situation in which the tissue should be rendered incapable of responding to the animal's own endogenous estrogens and, hence, unable to grow.

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Antagonism of Development and Growth of 7,12-Dimethylbenz(a)anthracene-induced Rat Mammary Tumors by the Antiestrogen U 23,469 and Effects on Estrogen and Progesterone Receptors

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