Antitumor Effects of Droloxifene, a New Antiestrogen Drug, against 7,12-Dimethylbenz(a)anthracene-Induced Mammary Tumors in Rats

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ABSTRACT—The antitumor effects of droloxifene (DROL, (E)-α-[p-[2-(dimethylamino)ethoxy]-phenyl]-α-ethyl-3-stilbenol), a new antiestrogen drug, on 7,12-dimethylbenz(a)anthracene (DMBA)-induced estrogen-dependent mammary tumors in rats were studied and compared with those of tamoxifen (TAM). Mammary tumors appeared from about 2 months after p.o. administration of DMBA to female rats, and all of them were estrogen receptor (ER) and progesterone receptor positive. DROL and TAM (p.o.) inhibited the growth of the tumors. Both drugs inhibited the binding of 125I-estradiol-17β to ER in the cytosol of the tumor in vitro, and the effect of DROL was much stronger than that of TAM. When 3H-estradiol-17β (3H-E2) was given s.c. to rats with the mammary tumors, 3H-E2 accumulated in the tumors, uteri and vaginas in which the ER levels are known to be high, but was low in the heart, in which the ER levels are normally low. DROL and TAM decreased the levels of 3H-E2 in the tumors, uteri and vaginas, but had no effect in the hearts. When DROL or TAM was given p.o. to rats daily for 7 consecutive days after administration of DMBA, they inhibited the induction of mammary tumors by DMBA. From these results, DROL inhibits the growth and the initiation of DMBA-induced mammary tumors by inhibiting the binding of estrogen to its receptor.

Some estrogen receptor (ER)-positive breast cancers are hormone-dependent and require estrogen for growth. Thus, breast cancer remission has been demonstrated following ablative endocrine therapy (1, 2). Antiestrogen drugs such as tamoxifen (TAM) are now widely used for endocrine treatment of breast cancers, and they provide successful management of hormone-dependent breast cancers (3–5). TAM inhibits the growth of ER-positive breast cancers by inhibiting the binding of estrogen to ER in the cells (6–8). However, ER-positive breast cancers are not always sensitive to TAM; and thus, efforts have been made to seek drugs with stronger antiestrogenic activity (9, 10). In a previous study, we showed that droloxifene (DROL, (E)-α-[p-[2-(dimethylamino)ethoxy]-phenyl]-α-ethyl-3-stilbenol), a new antiestrogen, had higher affinity to ER in human ER-positive MCF-7 breast cancer cells and rat uterus when compared with TAM (11). Huggins et al. were the first to induce hormone-dependent mammary adenocarcino-
mas in female Sprague-Dawley rats by administering the polycyclic hydrocarbon 7,12-dimethylbenz(a)anthracene (DMBA) (12). This experimental tumor has been widely used as a good model for evaluating the antitumor effects of various drugs and for predicting their clinical potential (13–17). Since TAM is known to inhibit the growth of DMBA-induced mammary carcinoma (15), we evaluated the effects of DROL on the tumors in rats and attempted to identify the mechanism of its action.

MATERIALS AND METHODS

Chemicals

DROL and TAM were provided by Klinge Pharma GmbH (Munich, FRG). The chemical structures of the drugs are shown in Fig. 1. Both drugs were used as citrate. DMBA was purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan) and estradiol-17β (E2). From Sigma Chemical Co. (St. Louis, MO, U.S.A.). 16α-125I-Estradiol-17β (81.4 TBq/mmol, 125I-E2) was purchased from Otsuka Pharmaceutical Co. (Tokyo, Japan); and (6, 7) 3H-estradiol-17β (2220 GBq/mmol, 3H-E2), 3H-R5020 (3.22 TBq/mmol) and R5020 were from New England Nuclear Research Products (Boston, MA, U.S.A.). For the in vivo tests, DROL and TAM were dissolved in dimethyl sulfoxide and diluted with the buffer.

Animals and tumor induction

Female Sprague-Dawley rats were purchased from Japan SLC, Inc. (Shizuoka, Japan). DMBA was given p.o. to rats (7 weeks old) in a dose of 20 mg. Palpable mammary tumors appeared from about 60 days, and the occurrence of the tumors gradually increased until approximately 90% of the rats had tumors about 100 days after DMBA administration.

Rats were ovariectomized under pentobarbital anesthesia to eliminate endogenous estrogen and progesterone prior to the experiments to measure the levels of ER and progesterone receptor (PgR), in vitro binding of estrogen to ER and in vivo incorporation of estrogen into organs. This is necessary because endogenous hormone competes with the exogenous hormone in binding studies.

Measurement of receptor levels

About 100 days after the administration of DMBA, 5 rats bearing mammary tumors (total of 17 tumors) were ovariectomized; and 4 days later, all the tumors were removed and homogenized in Tris-EDTA-dithiothreitol (TED) buffer (10 mM Tris/HCl, 1.5 mM EDTA, 0.5 mM dithiothreitol, 10% glycerol, pH 7.4). The homogenate was then centrifuged at 105,000 × g for 30 min to obtain the cytosol as the supernatant fraction. The cytosol was incubated with various concentrations of 125I-E2 with or without unlabelled E2 at 4°C.

Fig. 1. Structures of DROL and TAM.
for 16 hr. Dextran-coated charcoal (DCC) suspension was added to the cytosol to remove $^{125}$I-E2 and E2 unbound to ER (18), and the mixture was allowed to stand for 15 min. The charcoal was sedimented by centrifugation at 3,000 rpm for 20 min, and the radioactivity in the supernatant was counted using a γ-counter. Saturability of the binding sites ($B_{\text{max}}$) and the apparent dissociation constant ($K_d$) were calculated according to Scatchard analysis (19).

Additionally, by the same method, $B_{\text{max}}$ and $K_d$ values for PgR of each tumor were measured using $^3$H-R5020 and unlabelled R5020.

When the level of ER or PgR of the tumor was more than 5 fmol/mg protein each, the mammary tumor was considered as ER- or PgR-positive.

**Evaluation of antitumor effects on DMBA-induced mammary tumors**

DROL or TAM was given p.o. to the rats daily for 3 weeks from 92 days after administration of DMBA. The 2 perpendicular diameters of the tumors were measured once a week, and the tumor area was calculated as the product of the diameters. Relative tumor area was obtained using the formula: relative tumor area ($\%$) = (individual tumor area at a given time/initial individual tumor area) $\times$ 100.

**In vitro binding assay**

The cytosol of DMBA-induced tumor in rats was extracted by the method described above, and incubated with $^{125}$I-E2 ($5 \times 10^{-10}$ M) with or without unlabelled E2, and with DROL or TAM in TED buffer at 4°C for 16 hr. The DCC suspension was then added to the cytosol, and the mixture was centrifuged to sediment the charcoal. The radioactivity in the supernatant was counted.

**In vivo incorporation assay**

Ninety-four days after administration of DMBA, rats with mammary tumors were ovariectomized. Twenty-four hours later, DROL or TAM was given p.o. to the rats; and 1 hr later, $^3$H-E2 (1.1 MBq) in 0.1 ml of 5% ethanol-peanut oil was given s.c. The rats were killed 4 hr after the injection of $^3$H-E2; and their mammary tumors, uteri, vaginae and hearts were removed, weighed wet and solubilized in tissue solubilizer PROTOSOL$^R$ (NEN). The radioactivity in the tissue solubilized solution was counted, and the results were expressed as dpm/100 mg wet weight tissue.

**Evaluation of antitumor effects on the induction of mammary tumors by DMBA**

DROL or TAM was given p.o. to rats daily for 7 consecutive days from one day after the administration of DMBA. In the other groups, the rats were ovariectomized or sham-operated under pentobarbital anesthesia on the day after the administration of DMBA. The rats were inspected for the appearance of palpable mammary tumors at 2-week intervals for 20 weeks.

**Statistical analysis**

Analysis of variance was performed, and significance of differences was determined by the Dunnett or Tukey multiple comparison test or the Fisher exact test.

**RESULTS**

**Receptor assay**

ER and PgR levels were determined in the cytosol of all of the DMBA-induced mammary tumors in 5 rats by assessing the binding of $^{125}$I-E2 and $^3$H-R5020 to ER and PgR, respectively. Table 1 shows that the ER levels of the tumors ranged between 24 and 305 fmol/mg protein, and that their PgR levels were between 81 and 409 fmol/mg protein. The results suggested that the mammary tumors were ER and PgR positive.

**Antitumor effects on DMBA-induced mammary tumors**

Mammary tumors appeared in rats from about 60 days after DMBA was given p.o. We
examined the effects of DROL and TAM on the growth of the tumors. As shown in Fig. 2, the tumors grew steadily in the control group, but their growth was inhibited dose-dependently in the DROL groups or inhibited but not in a dose-dependent manner in the TAM groups.

In vitro binding affinities to ER in the cytosol of the tumor

Figure 3 shows that the binding of $^{125}$I-E$_2$ to ER in the cytosol of DMBA-induced rat mammary tumor was inhibited by E$_2$, DROL and TAM. The concentrations of E$_2$, DROL and TAM required to inhibit the binding by 50% were $1.8 \times 10^{-10}$, $2.3 \times 10^{-9}$ and $3.6 \times 10^{-7}$ M, respectively. DROL was about 100-fold stronger than TAM.

Inhibition of $^3$H-E$_2$ incorporation in the tumors in rats (in vivo)

DROL or TAM was given p.o. to ovariectomy rats with DMBA-induced mammary tumors; and 1 hr later, $^3$H-E$_2$ was given s.c. Figure 4 shows the levels of $^3$H-E$_2$ in 4 organs of the rats at 4 hr after the injection of $^3$H-E$_2$. In the control group, the levels were high in the mammary tumors, uteri and vaginae, but were very low in the hearts. DROL and TAM both significantly inhibited the accumulation of $^3$H-E$_2$ in the tumors, uteri and vaginae; and DROL was as active as TAM. However,
Fig. 2. Antitumor effects of DROL and TAM against DMBA-induced rat mammary tumors. DROL or TAM was given p.o. to the rats daily for 3 weeks from 92 days after p.o. administration of DMBA (20 mg), and the tumor area was calculated. Rats were used in groups of 8 (14 in the control group). Each point represents the mean and S.E. (A) DROL, ○: control, ●: 0.32 mg/kg, △: 1.0 mg/kg, ▲: 3.2 mg/kg; (B) TAM, ○: control, ●: 0.32 mg/kg, △: 1.0 mg/kg, ▲: 3.2 mg/kg. *: P < 0.05, **: P < 0.01, compared with the control group (Dunnett multiple test).
Fig. 3. In vitro binding affinities of DROL and TAM to ER in the cytosol of DMBA-induced rat mammary tumor. The cytosol was incubated at 4°C for 16 hr with $^{125}$I-E2 with or without DROL (●), TAM (△) or E2 (○). The binding affinities of the drugs were determined by the DCC method. All experiments were done in triplicate. Each point represents the mean and S.E.

Fig. 4. In vivo binding affinities of DROL and TAM to ER in DMBA-induced mammary tumors in rats. Rats with DMBA-induced mammary tumors were ovariectomized, and DROL or TAM was given p.o. to rats. One hour later, $^3$H-E2 was given s.c.; and 4 hr later, the rats were killed and their mammary tumors, uteri, vaginas and hearts were removed. The radioactivity was measured, and the results were expressed as dpm/100 mg wet weight tissue. Rats were used in groups of 3. Number of tumors in each group is shown in parentheses. Each column represents the mean and S.E. **: P < 0.01, compared with the control group (Dunnett multiple test). No significant difference between the groups treated with DROL and TAM in the same dose was observed (Tukey multiple test).
neither drug had an effect on the level of $^3$H-E$_2$ in the hearts.

**Antitumor effects on induction of mammary tumors by DMBA**

We examined the effects of DROL and TAM on the induction of mammary tumors by administration of DMBA. The results are shown in Table 2. In the control group, the number of rats with tumors increased gradually. In the DROL and TAM groups, 10 mg/kg of DROL and 1 mg/kg of TAM inhibited tumor initiation, but when the doses were reversed, neither drug had an inhibitory effect. Ovariectomy also had an inhibitory effect on tumor initiation, but sham-operation had no effect.

**DISCUSSION**

Mammary adenocarcinomas can be induced by p.o. administration of DMBA to Sprague-Dawley female rats. Since the tumors regress after ovariectomy or grow after injection of estrogen in rats, they are considered to be estrogen-dependent (12, 20). Generally, tumors transplanted in animals are used to evaluate the antitumor effects of drugs. However, the hormone responsiveness of transplantable mammary tumors seems to be affected by repeated experiments. Thus, the autochthonous mammary tumor induced by DMBA is the more appropriate model for studying the antitumor effects of drugs (13–17). In this paper, we studied the antitumor effects of DROL on the growth and initiation of DMBA-induced mammary tumors in female rats and compared them with those of TAM.

First we measured the levels of ER and PgR in DMBA-induced mammary tumors in rats. High levels of ER and PgR in the cytosol of all the tumors tested were observed. ER mediates estrogen action, and PgR is one of the estrogen-induced proteins in estrogen-dependent cells (21–23). A good correlation was reported between the levels of ER and PgR in tumors and hormone-dependency (24). From these results, we confirmed that DMBA-induced rat mammary tumors would provide a good hormone dependent experimental model.

DROL and TAM inhibited the growth of the mammary tumors in rats, but unlike TAM, the effect of DROL was dose-dependent. TAM was weaker in its antitumor effect

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of rats with tumors/No. of rats used</th>
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<tbody>
<tr>
<td></td>
<td>12 w*</td>
</tr>
<tr>
<td>Control</td>
<td>10</td>
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<tr>
<td>DROL 1 mg/kg</td>
<td>10</td>
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<tr>
<td>TAM 1</td>
<td>10</td>
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<tr>
<td>Ovariectomized</td>
<td>7</td>
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DMBA (20 mg) was given p.o. to rats; and from the next day, DROL or TAM was given p.o. daily for 7 consecutive days. In the other groups, the rats were ovariectomized or sham-operated on the day after the administration of DMBA. The rats were inspected for the appearance of palpable mammary tumors. *: Weeks after DMBA treatment. **: One rat died before 12 w. *: P < 0.05, compared with the control group. **: P < 0.05, compared with the sham-operated group (Fisher exact test).
in a dose of 3.2 mg/kg than in doses of 0.32 and 1 mg/kg. It is known that TAM has both estrogenic and antiestrogenic activities (4, 25, 26). Our previous experiment also showed that TAM had stronger estrogenic activity than DROL in increasing uterine weight in immature rats (11). The weak antitumor effect of TAM in a dose of 3.2 mg/kg might be dependent upon its relatively strong estrogenic activity.

In our in vitro experiment, DROL inhibited the binding of E2 to ER in the cytosol of the mammary tumor cells, with 100-fold higher affinity to ER than that of TAM. In our previous study, DROL showed higher affinity than TAM to ER in human ER-positive MCF-7 breast cancer cells and rat uterus (11). Our present findings are consistent with the results obtained in this study. In our in vivo experiment, high levels of $^3$H-E2 were observed in the mammary tumors, uteri and vaginas. This also is consistent with the reports that the tumors, uteri and vaginas contain high levels of ER and are estrogen-target tissues (15, 27). DROL and TAM significantly reduced the levels of $^3$H-E2 in the tumors and similarly reduced them in the uteri and vaginas in rats, suggesting that DROL has a high affinity to ER and that it inhibits the binding of E2 to ER in ER-positive mammary tumors. Therefore, DROL, like TAM (6–8), may exert its antitumor effects on ER-positive mammary tumors by inhibiting the binding of E2 to ER in the tumor cells. It was reported that in animals and humans, DROL was rapidly metabolized and its half-life was much shorter than that of TAM, and that TAM accumulated in blood with administrations, while DROL did not (28). The findings that DROL was almost as active in the affinity to ER as TAM in vivo, although DROL was more active than TAM in vitro may be due to differences in the metabolic mechanisms of both drugs in rats.

In general, the tumors in postmenopausal women are more sensitive to antiestrogen drugs than ones in premenopausal women. Thus, antiestrogen drugs are mainly used in the treatment of postmenopausal women with mammary tumors (5, 29), because the postmenopausal women have lower levels of endogenous estrogen than premenopausal women and antiestrogen drugs can easily inhibit the binding of a low level of endogenous estrogen to ER in the tumor cells. In the antitumor experiment, DROL and TAM would be more active in rats with low levels of endogenous estrogen than in normal rats. However, since TAM was also reported to show inhibition of estrogen-stimulated prolactin release (30) and of follicle-stimulating hormone-stimulated estrogen synthesis in animals (31), it is possible that the antitumor effects of DROL and TAM could be due to mechanisms other than their inhibitory effects on the binding of estrogen to ER in tumor cells. The detailed mechanism of action needs to be clarified.

In the last experiment, we studied the effects of DROL and TAM on the induction of mammary tumors by DMBA in rats. We showed that treatment with DROL at 10 mg/kg for 7 days starting the day after administration of DMBA reduced the incidence of tumors, but that DROL at 1 mg/kg was not effective. On the other hand, TAM at 1 mg/kg also reduced the incidence of tumors, but a high dose of TAM (10 mg/kg) was not effective. Ovariectomy abolished this induction, and estrogen restored the incidence of the tumors (20, 32–35). From these findings, estrogen seems to be essential for the initiation of mammary tumors by DMBA. Since ER was observed in mammary glands (36), DROL and TAM may inhibit the binding of estrogen to ER in mammary glands. Therefore, the inhibitory effects of DROL and TAM on the tumor induction may be due to their inhibiting effects on the binding of estrogen to ER in the mammary glands of rats. With the high dose of TAM, its lack of inhibitory effect might be related to its estrogenic activity. Furthermore, as described above, antiestrogens inhibit prolactin release and estrogen synthesis (30, 31). The involvement of these effects can not be ruled out as a mechanism for their inhibition of tumor induction by
DMBA in rats. However, further study along these lines is needed to reach a conclusion.

In this study, we demonstrated that DROL inhibits the growth of autochthonous ER-positive mammary tumors and reduces the incidence of DMBA-tumors in rats by inhibiting the binding of estrogen to ER. Our results suggest that DROL has clinical potential for use against ER-positive breast cancers.

REFERENCES