The Estrogenic and Antiestrogenic Activities of Droloxifene in Human Breast Cancers

Ikuo Kawamura, Tamotsu Mizota, Elizabeth Lacey, Yoshio Tanaka, Toshitaka Manda, Kyoichi Shimomura and Masanobu Kohsaka

Department of Pharmacology, Product Development Laboratories, Fujisawa Pharmaceutical Co., Ltd., 2-1-6, Kashima, Yodogawa-ku, Osaka 532, Japan

Received March 18, 1993 Accepted May 31, 1993

ABSTRACT—The effects of a new antiestrogen, droloxifene (DROL, (E)-α-[p-[2-(dimethylamino)ethoxy]-phenyl]-α‘-ethyl-3-stilbenol), on human breast cancer cells in vitro and in vivo were studied. Since phenol red has a binding affinity to the estrogen receptor (ER), we studied the activities of drugs in medium with or without this indicator. Estradiol-17β (E2) stimulated the growth of ER-positive breast cancer cells, MCF-7, ZR-75-1 and T-47D, in medium without phenol red, but not in medium containing this indicator. In medium without phenol red, DROL had no marked effects on the growth of MCF-7 and ZR-75-1, but slightly stimulated the growth of T-47D. Tamoxifen (TAM) stimulated the growth of these 3 cells. DROL dose-dependently inhibited the E2-induced stimulation of growth of these cells in medium without phenol red, but TAM inhibited the growth only at high concentrations. The growth of ER-negative breast cancer cells, MDA-MB-231, was not influenced by E2, DROL or TAM. DROL was more effective than TAM against ER-positive Br-10 breast carcinoma in nude mice, but neither drug had effects on ER-negative MX-1 breast carcinoma. These results suggest that DROL shows an antitumor effect on ER-positive breast cancers, being less estrogenic and more antiestrogenic than TAM.

Keywords: Antitumor, Antiestrogen drug, Droloxifene, Phenol red, Tumor (human breast)

The growth of some estrogen receptor (ER)-positive breast cancer cells is stimulated by estrogen (1, 2). The antiestrogen drug tamoxifen (TAM) exerts an antitumor effect on ER-positive breast cancers by inhibiting the binding of estrogen to the ER in the cells (3–5). In the clinic, TAM is a widely used first line treatment of ER-positive breast cancers and has achieved successful management of these cancers (6–8). However, ER-positive breast cancers are not always sensitive to TAM (9). TAM also shows estrogenic activity that can lead to side effects such as hot flush and vaginal bleeding (10, 11). Furthermore, it is possible that these estrogenic effects actually may stimulate the growth of ER-positive breast cancer cells. Efforts have been made to develop compounds with stronger antiestrogenic and less estrogenic effects than TAM. A new antiestrogen, droloxifene (DROL, (E)-α-[p-[2-(dimethylamino)ethoxy]-phenyl]-α‘-ethyl-3-stilbenol), is being developed as a derivative of TAM (12–14). In the previous study, we showed that DROL inhibited the binding of estrogen to the ER and had stronger antiestrogenic and less estrogenic effects than TAM in tests on uterine weight in rats (15).

Recently, cell lines established from human breast tumors have been widely used as models of estrogen-dependent or -independent breast cancers in both in vitro and in vivo tests to study the antitumor effect of antiestrogens (16–19). Phenol red is commonly used as a pH indicator in the culture media. Since this indicator shows binding affinity to the ER in the cells, has an estrogenic effect, and also stimulates the growth of estrogen-dependent breast cancer cells (20–23), it is likely that its removal from the media may be necessary to evaluate the real effects of antiestrogen on the growth of estrogen-dependent breast cancer cells.

In this study, we examined the effect of DROL on the growth of in vitro cultured human breast cancer cells in medium without phenol red, and also studied the in vivo antitumor effect of DROL on human breast carcinomas transplanted in nude mice.
MATERIALS AND METHODS

Drugs

DROL and TAM were provided by Klinge Pharma GmbH (Munich, FRO). Both drugs were used as the citrate salt. Estradiol-17β (E2), bovine insulin, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) and Dulbecco’s modified Eagle’s medium and Ham’s nutrient mixture F-12 (DME/F-12) without phenol red were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco’s modification of Eagle’s medium (DMEM) and penicillin-streptomycin solution were from Flow Laboratories (Irvine, Scotland). The DMEM medium contained 42 µM phenol red. Fetal calf serum (FCS) was from Gibco Laboratories (Grand Island, NY, USA). Dextran-coated charcoal-treated FCS (DCC-FCS) was prepared as described before (15). E.P. Hormone Depot® was purchased from Teikoku Zoki Pharmaceutical Co. (Tokyo). Hydroxyprogesterone caproate (50 mg) and estradiol dipropionate (1 mg) were included in 1 ml of this hormone solution.

Cultured human breast cancer cells

The 4 kinds of cultured human breast cancer cells (MCF-7, ZR-75-1, T-47D and MDA-MB-231) were maintained in DMEM supplemented with 10% FCS, 0.1 units/ml insulin and 50 units/ml penicillin-50 µg/ml streptomycin. Cells growing as monolayers were kept at 37°C in a humid atmosphere in the presence of 5% CO2.

Cell growth tests

MCF-7 (2 x 10³ cells), MDA-MB-231 (2 x 10³ cells), ZR-75-1 (4 x 10³ cells), and T-47D (6 x 10³ cells) were incubated in 100 µl of medium containing 5% DCC-FCS in 96-well microtiter plates for 24 hr. The medium was replaced by a fresh one containing drugs and the cultures were incubated for 7 days. The cell growth was determined by the MTT assay (15, 24). All experiments were performed in triplicate. The effect on cell growth was expressed as a percentage of the control level. DROL, TAM and E2 were dissolved in dimethyl sulfoxide (DMSO) and diluted with the medium (DMSO: final concentration 0.1% or less).

Animals

Female BALB/c nu/nu and MCH(AF)-nu mice were purchased from Clea Japan, Inc. (Tokyo). The mice were kept in conditions of constant temperature and humidity and fed a standard diet and water ad libitum.

In vivo tests against human breast carcinomas

Br-10 and MX-1 human breast carcinomas were maintained subcutaneously (s.c.) by serial passage in BALB/c nu/nu mice.

In the tests, fragments (3 x 3 x 3 mm) of Br-10 or MX-1 were implanted s.c. into the left flank of MCH(AF)-nu or BALB/c nu/nu mice, respectively. E.P. Hormone Depot®, containing hydroxyprogesterone caproate and estradiol dipropionate, at a volume of 0.1 ml/body was given once intramuscularly (i.m.) to the mice on the same day as implantation with Br-10 (25–27). The tumor weight as derived from caliper measurements of the length and width of the tumors was calculated as follows:

Tumor weight (mg) = 1/2 x a x b²

where a represents the length and b represents the width (mm). Drug efficacy was based on the ratio of the percentage of growth inhibition of the drug-treated group to that of the control group.

\[
\text{Growth inhibition (\%)} = \left(1 - \frac{Tn/To}{Cn/Co}\right) \times 100
\]

Co: Mean tumor weight initially in the control group
Cn: Mean tumor weight on a given day in the control group
To: Mean tumor weight initially in the drug-treated group
Tn: Mean tumor weight on a given day in the drug-treated group

DROL and TAM were dissolved in DMSO and diluted with sesame oil (DMSO: final concentration 10%). The drugs were given to the mice at a volume of 5 ml/kg.

Statistical analysis

Analysis of variance was performed and the Tukey test was used to determine significance of differences.

RESULTS

Effect of E₂ on the growth of breast cancer cells

To study the effects of E₂ on the growth of human breast cancer cells, the breast cancer cells were cultured with E₂ in medium with or without phenol red for 7 days, and the cell growth was determined by MTT assay. The results are shown in Figs. 1 and 2. E₂ stimulated the growth of MCF-7, ZR-75-1 and T-47D in medium without phenol red, but not in medium with phenol red. The growth of the ER-negative breast cancer cells, MDA-MB-231, was not influenced by E₂ in either media.

We also examined the effects of phenol red in the medium on the growth of the breast cancer cells. When the cancer cells were incubated for 7 days, the growth of MCF-7 in the medium with phenol red was about twice as high as that in the medium without it, and the growth of ZR-75-1, T-47D or MDA-MB-231 was similar in both types of media (Table 1).
Fig. 1. Effects of E₂ on the growth of 4 human breast cancer cells, MCF-7 (A), ZR-75-1 (B), T-47D (C) and MDA-MB-231 (D), in medium with phenol red. Cells were incubated with the indicated concentrations of E₂ for 7 days. Cell growth was determined by MTT assay; results are expressed as a percentage of the control. All experiments were performed in triplicate. Each column represents the mean and S.E.

Fig. 2. Effects of E₂ on the growth of 4 human breast cancer cells, MCF-7 (A), ZR-75-1 (B), T-47D (C) and MDA-MB-231 (D), in medium without phenol red. The method was described in the legend of Fig. 1. All experiments were performed in triplicate. Each column represents the mean and S.E. **: P<0.01, compared with the control group (Tukey test).
Table 1. The growth of 4 human breast cancer cells

| Cell growth | Phenol red (−) | Phenol red (+) |
|-------------|----------------|----------------|
| MCF-7       | 0.281 ± 0.015  | 0.493 ± 0.037  |
| ZR-75-1     | 0.278 ± 0.009  | 0.349 ± 0.016  |
| T-47D       | 0.258 ± 0.003  | 0.263 ± 0.014  |
| MDA-MB-231  | 0.384 ± 0.011  | 0.337 ± 0.022  |

Cells were cultured for 7 days in medium with or without phenol red. Cell growth was determined by MTT assay. Results are expressed as the mean and S.E. of the optical density (OD_{570nm}) values of the triplicate wells.

Estrogenic effect on breast cancer cells

The effects of DROL and TAM on the growth of human breast cancer cells were studied in the medium without phenol red. As shown in Fig. 3, TAM at concentrations of $1 \times 10^{-10}$ and $1 \times 10^{-9}$ M significantly stimulated the growth of MCF-7, but DROL did not stimulate the growth at concentrations between $1 \times 10^{-11}$ and $1 \times 10^{-6}$ M. TAM was more effective than DROL in stimulating the growth of MCF-7. DROL slightly stimulated the growth of ZR-75-1 at concentrations of $1 \times 10^{-9}$ M or less, but not at concentrations between $1 \times 10^{-8}$ and $1 \times 10^{-6}$ M. TAM stimulated the growth of ZR-75-1 at all concentrations tested. Both drugs slightly stimulated the growth of T-47D, and DROL was almost as active as TAM. The growth of MDA-MB-231 was not affected by DROL and TAM at concentrations up to $1 \times 10^{-6}$ M. Both drugs showed non-specific toxic effects on all of the cells tested at a concentration of $1 \times 10^{-5}$ M.

Antiestrogenic effect on breast cancer cells

The antiestrogenic effects of DROL and TAM on the stimulation of growth of human breast cancer cells induced by E2 in medium without phenol red were studied. As shown in Fig. 4, the E2-induced stimulation of the
growth of MCF-7, ZR-75-1 and T-47D was dose-dependently inhibited by DROL. TAM inhibited the growth of MCF-7 and T-47D only at the concentration of $1 \times 10^{-6}$ M, but did not inhibit the growth of ZR-75-1. The growth of MDA-MB-231 was not affected by DROL or TAM at concentrations up to $1 \times 10^{-6}$ M. Both drugs also showed non-specific toxic effects on all of the cells at a concentration of $1 \times 10^{-5}$ M.

**Antitumor effects on human breast carcinomas in mice**

The antitumor effects of DROL and TAM on human ER-positive Br-10 and ER-negative MX-1 breast carcinomas transplanted in nude mice were studied. The drug was given i.m. to mice with Br-10 three times a week for 4 weeks beginning 32 days after tumor implantation. As shown in Fig. 5, DROL in doses of 10 and 32 mg/kg and TAM in the dose of 32 mg/kg inhibited the growth of Br-10, but the effects of both drugs were not significant. The highest growth inhibition ratios (%) were 51 and 53 for 10 mg/kg and 32 mg/kg of DROL, respectively, and 50 for 32 mg/kg of TAM, but the other doses of both drugs showed less than 49% growth inhibition in this ratio. In the case of MX-1, drug was given i.m. three times a week for 4 weeks beginning 10 days after tumor implantation. As shown in Fig. 6, neither drug affected the growth of MX-1.

**DISCUSSION**

Phenol red, a pH indicator dye, is widely used in culture media. As this indicator shows binding affinity to the ER and also exerts a weak estrogenic effect and stimulates the growth of estrogen-dependent breast cancer cells (20, 21, 23), we first compared the effects of phenol red in medium on the growth of ER-positive breast cancer cells. In medium containing phenol red, the growth of MCF-7 was stimulated, but not those of ZR-75-1 and T-47D. Moreover, the addition of E2 stimulated the growth of MCF-7 threefold, while the growths of ZR-75-1 and T-47D were each stimulated twofold in medium without
phenol red. These results suggest that there are differences in the response of ER-positive breast cancer to estrogenic stimulation, and that ZR-75-1 and T-47D are less sensitive than MCF-7. A phenol red concentration of 42 μM may be sufficient to stimulate the growth of MCF-7, but not enough to stimulate the growth of ZR-75-1 and T-47D; higher concentrations of phenol red may stimulate the growth of the two latter ER-positive breast cancers. On the other hand, E2 did not stimulate the growth of the 3 ER-positive breast cancer cells in medium with phenol red, although E2 is known to show the strongest binding affinity to ER and to stimulate the growth of ER-positive breast cancers (1, 2). A possible hypothesis might be that phenol red in the medium may inhibit the binding of E2 to ER in the cells, or that the binding of phenol red to sites other than ER may inhibit the action of E2 in the cells. The influence of phenol red on the action of E2 is suggested to be not so simple, and its detailed mechanism needs to be clarified. Thus, the evaluation of the effects of drugs on the growth of ER-positive breast cancer cells in culture medium without phenol red may give us the real estrogenic effects.

In clinical studies, TAM blood levels after single and continuous administration were found to be in the order of $10^{-7}$ M and $10^{-6}$ M for the $C_{max}$ and the steady state, respectively (28, 29). DROL blood levels after continuous administration were in the order of $10^{-7}$ M (30). We compared the estrogenic or antiestrogenic effects of both drugs at concentrations between $1 \times 10^{-8}$ and $1 \times 10^{-6}$ M, the blood levels of both drugs obtained in clinical studies. In medium without phenol red, TAM stimulated the growth of the 3 ER-positive human breast cancer cells, but growth was only slightly or not at all stimulated by DROL. The results suggest that the estrogenic effect of DROL is much weaker than that of TAM. DROL inhibited the E2-induced stimulation of the growth of the 3 ER-positive human breast cancer cells, but TAM inhibited it only in high concentrations. The results suggest that DROL has stronger antiestrogenic activity than TAM. In the previous study, we showed that DROL was more active than TAM in decreasing the uterine weight of mature rats and E2-treated immature rats, and that DROL was inactive in increasing the uterine weight of immature rats, while TAM was significantly active (15). The results in this study are consistent with our previous findings using the index of rat uterine weight. Löser et al. also reported the same findings on DROL in uterotropic and anti-uterotropic tests in rats (13). On the other hand, the growth of ER-negative human breast cancer cells, MDA-MB-231, was not influenced by E2, DROL or TAM. From these results, E2, DROL and TAM may exert their effects on ER-positive human breast cancer cells by mediation of ER.

In the in vivo experiments, we examined the antitumor effects of DROL and TAM on human breast carcinomas Br-10 and MX-1 in nude mice, and selected the i.m. route...
of administration, since the AUC\textsubscript{0–\infty} of DROL after i.m. dosing was higher than that after oral dosing (data not shown). In the experiment against ER-positive Br-10 breast carcinoma, we used MCH(AF)-nu mice, since E.P. Hormone Depot\textsuperscript{®} was required to stimulate the growth of Br-10 in nude mice (26), and these mice did not die after the administration of the hormone, while some of the BALB/c nu/nu mice died. DROL and TAM inhibited the growth of Br-10 breast carcinoma, and DROL was slightly more effective than TAM. The weaker antitumor effect of TAM might be due to its lower antiestrogenic and stronger estrogenic activities than DROL. As mentioned above, we showed that DROL has less estrogenic effect than TAM on the uterine growth in rats (15), suggesting that DROL has less side effects derived from the estrogenic activity than TAM. In this study, we showed that TAM stimulates the growth of ER-positive breast cancer cells more than DROL. It is possible that the estrogenic activity of TAM might weaken the antitumor activity. From these results, it is suggested that DROL has stronger antitumor activity against ER-positive breast tumors than TAM. Against ER-negative breast carcinoma MX-1 transplanted into mice, neither drug was effective.

In summary, DROL showed a strong antitumor effect against ER-positive breast carcinomas with strong antiestrogenic and lower estrogenic activities, suggesting the clinical benefits of using DROL.

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