Blockade of the Stimulatory Effect of Estrogens, OH-Tamoxifen, OH-Toremifene, Droloxifene, and Raloxifene on Alkaline Phosphatase Activity by the Antiestrogen EM-800 in Human Endometrial Adenocarcinoma Ishikawa Cells[1]

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ABSTRACT

Although temporary benefits of tamoxifen therapy are observed in up to 40% of women with breast cancer, this compound, which is known to possess mixed estrogenic and antiestrogenic activities, has been associated with increased risk of endometrial carcinoma. This study compares the effects of the novel nonsteroidal pure antiestrogen EM-800 and related compounds with those of a series of antiestrogens on the estrogen-sensitive alkaline phosphatase (AP) activity in human endometrial adenocarcinoma Ishikawa cells. Exposure to increasing concentrations of up to 1000 nM EM-800 or its active metabolite EM-652 alone failed to affect basal AP activity. In contrast, incubation with 10 nM (Z)-4-OH-tamoxifen, (Z)-4-OH-toremifene, droloxifene, or raloxifene increased the value of this estrogen-sensitive parameter by 3.3-, 3.5-, 2.2-, and 1.6-fold, respectively, a stimulatory effect that was completely reversed by simultaneous exposure to 30 nM EM-800. Moreover, the stimulation of AP activity induced by 1 nM 17β-estradiol was completely reversed by EM-800, EM-652, or ICI-182780, at the IC_{50} value of 1.98 $\pm$ 0.23, 1.01 $\pm$ 0.16, and 5.64 $\pm$ 0.59 nM, respectively, whereas the partial blockade exerted by (Z)-4-OH-tamoxifen, (Z)-4-OH-toremifene, or raloxifene was observed at IC_{50} values of 13.5 $\pm$ 3.80, 41.0 $\pm$ 7.2, and 3.74 $\pm$ 0.43 nM, respectively. Thus, as assessed by their activity in the human Ishikawa endometrial carcinoma cells, EM-800 and EM-652 are the most potent known antiestrogens in Ishikawa cells, and, most importantly, they are devoid of the estrogenic activity observed in these human endometrial cancer cells with (Z)-4-OH-tamoxifen, (Z)-4-OH-toremifene, droloxifene, and raloxifene.

INTRODUCTION

Estrogens are well known to play a predominant role in the development and growth of human breast cancer. Because removal of estrogens from all exogenous and endogenous sources is not feasible, especially because of the important contribution of the adrenals in women (1), major efforts have been devoted to the development of inhibitors of estrogen biosynthesis and action (2–4).

Tamoxifen, the only antiestrogen widely available for the treatment of women with breast cancer, has an efficacy comparable to that achieved with ablative and additive therapies (5). This compound, however, is known to possess mixed estrogenic and antiestrogenic activities that are highly species, tissue, cell, and even gene specific (Refs. 2 and 6–9 and references therein). In this context, although temporary benefits of tamoxifen are observed on breast cancer in up to 40% of women, the use of this compound has been associated with an increased incidence of endometrial carcinoma in the same women (Refs. 10–12 and references therein), suggesting an effect related to the estrogenic activity of tamoxifen.

Because data suggest that continuous long-term tamoxifen therapy is preferable to its usual short-term use (5), and studies are already in progress on the long-term administration of tamoxifen to prevent breast cancer (13), it becomes important to make available a pure antiestrogen that, due to its lack of estrogenic activity, should theoretically be more efficient than tamoxifen in treating breast cancer while simultaneously eliminating the risk of developing uterine carcinoma during its long-term use. This study compares the effect of EM-800 or its active metabolite, EM-652, with those of OH-tamoxifen, OH-toremifene, droloxifene, raloxifene, and ICI-182780 (14–18) on estrogen-sensitive AP^3 activity in human endometrial carcinoma Ishikawa cells. AP activity is well known to be stimulated by estrogens, whereas the other steroids, namely androgens, progestins, mineralocorticoids, or glucocorticoids, have no effect on this parameter in Ishikawa cells (19).

MATERIALS AND METHODS

Chemicals. E_2 was obtained from Steraloids (Wilton, NH). All media and supplements for cell culture were from Sigma Chemical Co. (St. Louis, MO), except FBS, which was from Hyclone (Logan, UT). EM-800, its ($-$) enantiomer, EM-776, as well as its active metabolite, EM-652, and the antiestrogens ICI-182780, (Z)-4-OH-tamoxifen, (Z)-4-OH-toremifene, and droloxifene were synthesized in the medicinal chemistry division of our laboratory (Fig. 1). Toremifene was kindly provided by Schering Plough (Kenilworth, NJ), whereas raloxifene [previously designated as keoxifene or LY156758 (17)] was originally a gift from Dr. J. A. Clemens (Lilly Research Laboratories, Indianapolis, IN) and was later synthesized in our laboratory.

Maintenance of Stock Cell Cultures. The human Ishikawa cell line derived from a well-differentiated endometrial adenocarcinoma (20) was kindly provided by Dr. Erlio Gurpide (Mount Sinai Medical Center, New York, NY). The Ishikawa cells were routinely maintained in Eagle's MEM containing 5% (v/v) FBS and supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, and 1 mM sodium pyruvate. Cells were plated in Falcon T75 flasks at a density of 1.5 × 10^6 cells at 37°C.

Cell Culture Experiments. Twenty-four h before the start of an experiment, Ishikawa cells near confluence were cultured in an EFBM consisting of a 1:1 (v/v) mixture of phenol red-free Ham's F-12 medium and DMEM, all supplements mentioned above, and 5% FBS treated twice with dextran-coated charcoal to remove endogenous steroids. Cells were then harvested with 0.1% pancreatin (Sigma) in 0.25 mM HEPES, resuspended in EFBM, plated in Falcon 96-well flat-bottomed microtiter plates at a density of 2.5 × 10^4 cells/well in a volume of 100 μl, and allowed to adhere to the surface of the plates for 24 h. Thereafter, medium was replaced with fresh EFBM containing the indicated concentrations of compounds in a final volume of 150 μl. Cells were incubated for 5 days, with a medium change after 48 h.

AP activity was measured as described previously (19). Plates were monitored at 405 nm in an ELISA plate reader (Bio-Rad; model 2550 EIA reader). Dose-response curves and EC_{50} as well as IC_{50} values were calculated using a weighted iterative nonlinear squares regression (21).

[3] The abbreviations used are: AP, alkaline phosphatase; E_2, 17β-estradiol; FBS, fetal bovine serum; EFBM, estrogen-free basal medium.
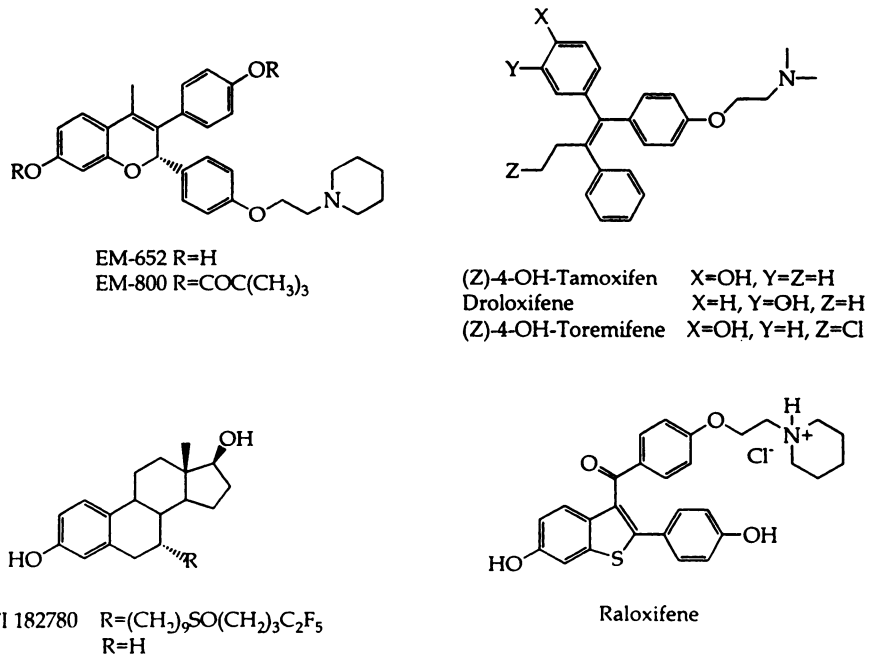
Fig. 1. Chemical structure of EM-652, EM-800, (Z)-4-OH-tamoxifen, (Z)-4-OH-toremifene, droloxifene, raloxifene (LY156758), ICI-182780, and E_2.

EM-652 R=H
EM-800 R=COC(CH₃)₃

(Z)-4-OH-Tamoxifen   X=OH, Y=Z=H
Droloxifene   X=H, Y=OH, Z=H
(Z)-4-OH-Toremifene   X=OH, Y=H, Z=Cl

ICI 182780   R=(CH₂)₉SO(CH₂)₃C₂F₅
E₂   R=H

Raloxifene

RESULTS

Incubation of human endometrial adenocarcinoma Ishikawa cells for 5 days with increasing concentrations of E_2 caused a maximal 15-fold increase in AP activity, with an EC_{50} value achieved at 9.77 ± 0.67 pM E_2 (data not shown). Exposure of Ishikawa cells to increasing concentrations (0.01–1000 nM) of the novel nonsteroidal antiestrogen EM-800 or its active metabolite EM-652 as well as of the steroidal antiestrogen ICI-182780 had no stimulatory effect on basal estrogen-sensitive AP activity (Fig. 2). It can also be seen in Fig. 2 that the marked stimulatory effect induced by 1 nM E_2 was competitively and completely reversed by EM-800, EM-652, and ICI-182780, with their potent inhibitory action of these antiestrogens

exerted at IC_{50} values of 1.98 ± 0.23, 1.01 ± 0.16, and 5.64 ± 0.59 nM, respectively.

On the other hand, incubation of Ishikawa cells for 5 days with increasing concentrations of (Z)-4-OH-tamoxifen caused a maximal 3.7-fold increase in AP activity, with an EC_{50} value achieved at 0.15 ± 0.02 nM, whereas (Z)-4-OH-toremifene caused a similar stimulatory effect on this estrogen-sensitive parameter at an EC_{50} value of 0.30 ± 0.05 nM (Fig. 3). We have also observed that exposure to droloxifene increased AP activity to similar levels at an EC_{50} value of 4.0 ± 0.39 nM (Fig. 2B). Moreover, as illustrated in Fig. 3, exposure to 0.1, 1, 10, and 100 nM raloxifene increased AP activity by 3.0-, 2.5-, 2.3-, and 2.1-fold, respectively. It can also be seen in this figure that
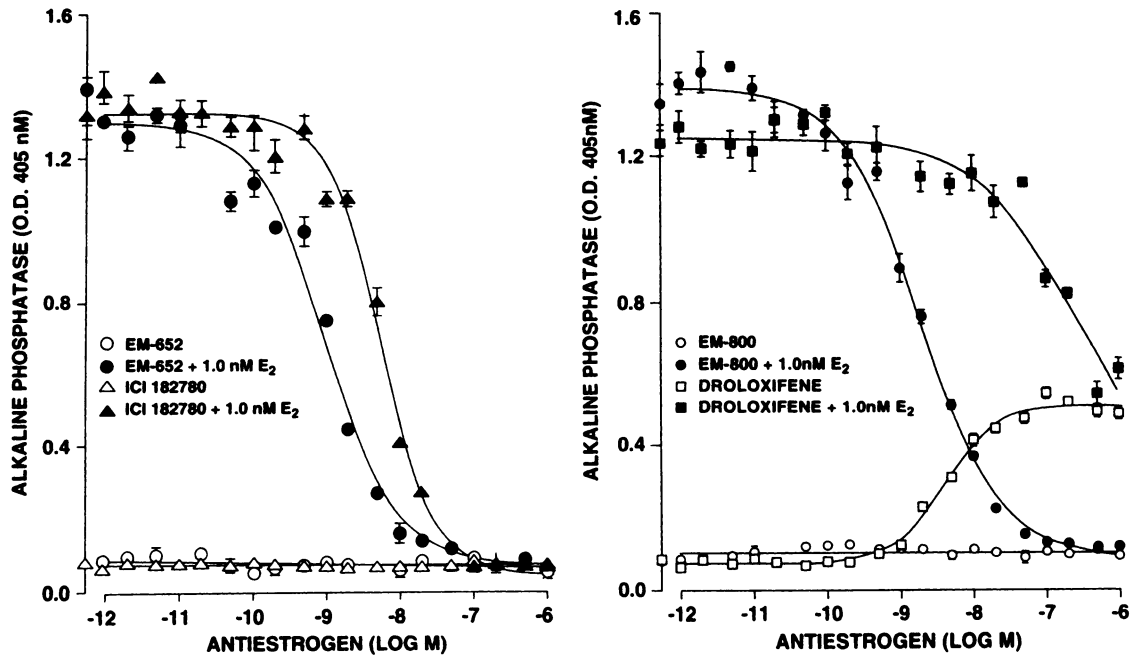

Fig. 2. Effect of increasing concentrations of the novel nonsteroidal antiestrogen EM-800, its active metabolite, EM-652, droloxifene, and the steroidal antiestrogen ICI-182780 on AP activity in human Ishikawa endometrial carcinoma cells. AP activity was measured after a 5-day exposure to increasing concentrations of the indicated compounds in the presence or absence of 1.0 nM E_2. Data are expressed as the means ± SE of four wells. When SE overlaps with the symbol used, only the symbol is shown.
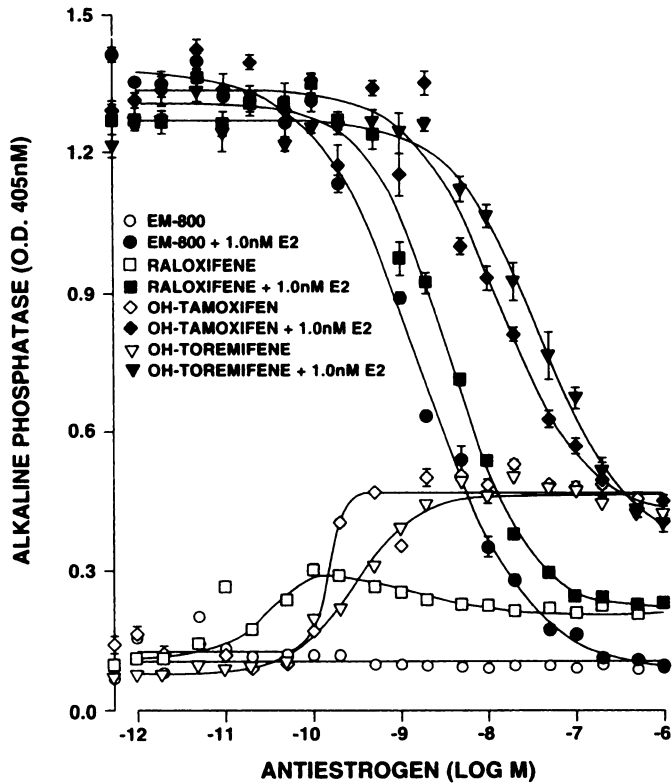
Fig. 3. Effect of increasing concentrations of EM-800, (Z)-4-OH-tamoxifen, (Z)-4-OH-toremifene, and raloxifene on AP activity in human Ishikawa cells. AP activity was measured after a 5-day exposure to increasing concentrations of the indicated compounds in the presence or absence of 1.0 nM E_2. Data are expressed as the means $\pm$ SE of four wells. When SE overlaps with the symbol used, only the symbol is shown.

the marked stimulatory effect exerted by 1 nM E_2 was competitively but not completely reversed by (Z)-4-OH-tamoxifen, (Z)-4-OH-toremifene, and raloxifene, with their partial inhibitory action exerted at IC_{50} values of 13.5 $\pm$ 3.8, 41.0 $\pm$ 7.2, and 3.74 $\pm$ 0.43 nM, respectively, whereas the E_2-induced AP activity was completely blocked by a simultaneous exposure to EM-800 at an IC_{50} value of 1.73 $\pm$ 0.19 nM.

A direct comparison of the estrogen-like activity of these antiestrogens can best be made in Fig. 4. Incubation with 3 nM (Z)-4-OH-tamoxifen, (Z)-4-OH-toremifene, droloxifene, or raloxifene increased the value of AP activity by 3.3-, 3.6-, 1.3-, and 1.7-fold, respectively, whereas exposure to 10 nM of these compounds increased the value of the same parameter by 3.3-, 3.5-, 2.2-, and 1.6-fold, respectively. The complete blockade of the stimulatory effect of all these antiestrogens on AP activity by simultaneous exposure to EM-800 strongly supports the suggestion that the stimulatory effect of (Z)-4-OH-tamoxifen, (Z)-4-OH-toremifene, droloxifene, and raloxifene on this parameter is mediated through the estrogen receptor (Fig. 4).

DISCUSSION

The present data clearly demonstrate that the novel nonsteroidal antiestrogen EM-800 and its active metabolite, EM-652, exert pure antagonistic effects while being the most potent of the compounds tested on E_2-induced AP activity in human endometrial adenocarcinoma Ishikawa cells. In contrast to EM-800 and EM-652, (Z)-4-OH-tamoxifen, (Z)-4-OH-toremifene, droloxifene, and raloxifene exert a stimulatory effect on this estrogen-sensitive parameter, an effect that can be completely blocked by simultaneous exposure to the antiestrogen EM-800, thus strongly supporting the suggestion that the

stimulatory effect of these antiestrogens is mediated through activation of the estrogen receptor.

The recent observations show that the estrogenic as well as antiestrogenic activity of compounds is not only species specific but is also tissue, cell, and even gene specific (Refs. 6, 8, and 9 and references therein; Ref. 22), making it imperative to test potential antiestrogens in the same cells that are targets for therapy. In this regard, this study shows that measurement of AP activity in Ishikawa cells *in vitro* can be used as a precise, sensitive, and easy-to-perform assay of estrogenic and/or antiestrogenic activity of compounds in human uterine cells.

The appearance of uterine carcinoma in women treated with tamoxifen (Refs. 11 and 12 and references therein) is not surprising, because tamoxifen has been shown to stimulate the growth of two human endometrial tumors implanted in nude mice (6, 23, 24) as well as *in vitro* (25–27). Furthermore, (Z)-4-OH-tamoxifen has been shown to be potent and sometimes even more potent than E_2 itself in stimulating progesterone receptors in the human Ishikawa endometrial cell line (27). It should be added that the relationship between estrogens and endometrial carcinoma is well known (28–30). As further support to the data obtained in human endometrial carcinoma, the potent stimulatory effect of tamoxifen on estrogen-sensitive parameters in the normal uterus is also well known in the mouse, rat, and hamster (2, 31, 32). Therefore, it seems that the estrogenic activity of tamoxifen in the uterus is common to all parameters and species studied thus far. Thus, it is not surprising that the two other tamoxifen-related compounds, namely, (Z)-4-OH-toremifene and droloxifene, also possess an estrogenic activity in human Ishikawa cells.

A stimulatory effect of tamoxifen has been demonstrated repeatedly not only in human uterine cells but also in human breast cancer cells *in vitro* (7, 22, 33–35) as well as in *in vivo* nude mice (6). (Z)-4-OH-tamoxifen has also been shown to exert estrogenic activity on pS2 mRNA levels in MCF-7 cells (22). Moreover, both (Z)-4-OH-tamoxifen and the steroidal antiestrogen ICI-164384 stimulated CAT activity in MCF-7 cells transfected with a pS2-tk-CAT fusion gene (22). In addition, subnanomolar concentrations of raloxifene had a significant estrogenic stimulatory effect on the cell growth of ZR-75-1 human breast cancer cells (7).

The relevance of our observation concerning the estrogenic activity of (Z)-4-OH-tamoxifen also pertains to the observation that the average serum concentration of (Z)-4-OH-tamoxifen in women treated
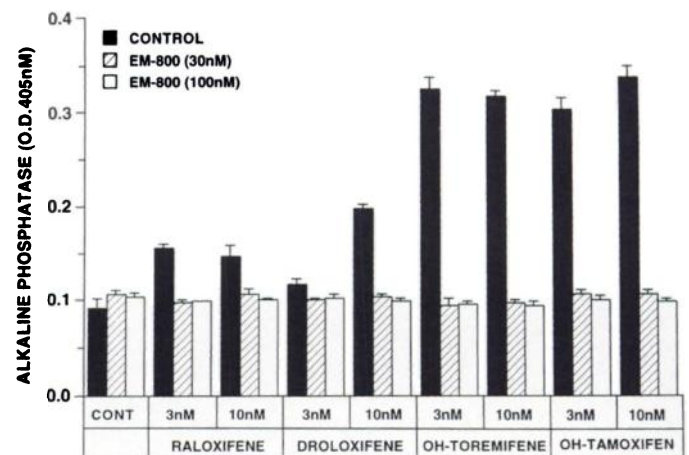

Fig. 4. Blockade of the stimulatory effect of (Z)-4-OH-tamoxifen, (Z)-4-OH-toremifene, droloxifene, and raloxifene on AP activity by the antiestrogen EM-800 in human Ishikawa carcinoma cells. AP activity was measured after a 5-day exposure to 3 or 10 nM of the indicated compounds in the presence or absence of 30 or 100 nM EM-800. Data are expressed as the means $\pm$ SD of eight wells, with the exception of the control groups, where data were obtained from 16 wells.

chronically with tamoxifen for breast cancer ranges from 2.0–10 nM (36), indicating that such circulating levels of the antiestrogen should have a near-maximal estrogenic effect in endometrial cells.

The consequence of the partial agonistic activity of tamoxifen is that "complete blockade of the action of estrogens cannot be achieved with tamoxifen" (3). Thus, it is reasonable to expect that the availability of a pure antiestrogen, in addition to avoiding the risk of inducing endometrial carcinoma, should show significant benefits over tamoxifen in the treatment of breast cancer. In this regard, we have synthesized EM-800 and its active metabolite, EM-652, which show a 1.5–3.0 times higher activity than E_2 and diethylstilbestrol to displace $[^3H]E_2$ from the estrogen receptor in human breast cancer and normal uterine tissue (37). EM-800 and EM-652 are potent pure antiestrogens in the human breast cancer models studied both *in vitro* and *in vivo* in nude mice (37–39). Moreover, EM-800 administered by the oral route is 2–3-fold more potent than ICI-182780 administered s.c. (40). Clinical trials are in progress to assess the safety and tolerance of EM-800 as well as to obtain information on the efficacy of EM-800 in patients in progression after a positive response to treatment with tamoxifen.

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