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CHARACTERIZATION OF THE EFFECTS OF THE NOVEL NON-STEROIDAL ANTIESTROGEN EM-800 ON BASAL AND ESTROGEN-INDUCED PROLIFERATION OF T-47D, ZR-75-1 AND MCF-7 HUMAN BREAST CANCER CELLS *IN VITRO*

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Since estrogens play a predominant role in the development and growth of human breast cancer, antiestrogens represent a logical approach to the treatment of this disease. The present study compares the effects of the novel non-steroidal anti-estrogen EM-800 and related compounds with those of a series of anti-estrogens on basal and 17β-estradiol (E_2)-induced cell proliferation in human breast cancer cell lines. In the absence of added E_2, EM-800 and related compounds failed to change basal cell proliferation, thus showing the absence of intrinsic estrogenic activity in the ER-positive T-47D, ZR-75-1 and MCF-7 cell lines. The stimulation of T-47D cell proliferation induced by 0.1 nM E_2 was competitively blocked by a simultaneous incubation with EM-652, EM-800, OH-tamoxifen, OH-toremifene, ICI 182780, ICI 164384, droloxifene, tamoxifen and toremifene at apparent Ki values of 0.015, 0.011–0.017, 0.040–0.054, 0.043, 0.044, 0.243 and 0.735 nM, approx. 10 nM and >10 nM, respectively. Similar data were obtained in ZR-75-1 and/or MCF-7 cells. Moreover, EM-652 was 6-fold more potent than OH-Tamoxifen in inhibiting the proportion of cycling MCF-7 cells. Our data show that EM-800 and EM-652 are the most potent known antiestrogens in human breast cancer cells *in vitro* and that they are devoid of the estrogenic activity of OH-tamoxifen and droloxifene suggested by stimulation of cell growth in the absence of estrogens in ZR-75-1 and MCF-7 cells. *Int. J. Cancer* 73:104–112, 1997.
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Breast cancer is the most frequent cancer in women, the lifetime odds of developing it being 1:8. It is predicted that 180,200 new cases of female breast cancer will be diagnosed in the United States in 1997, while 43,900 women are expected to die from the disease during the same period (Parker *et al.,* 1997). Among all factors, estrogens are well recognized to play the predominant role in breast cancer development and growth. Unfortunately, the existing surgical or medical ablative procedures do not permit complete elimination of estrogens in women, especially due to the important contribution of the adrenals, which secrete high levels of dehydroepiandrosterone (DHEA) and DHEA-sulfate; these are converted into estrogens in peripheral target tissues (Labrie, 1991). Considerable attention has thus focused on the mechanisms of action of estrogens and especially on the development of blockers of estrogen biosynthesis and action (Labrie *et al.,* 1992; Wakeling, 1993, Jordan, 1994).

Since the first step in the action of estrogens in target tissues is binding to the estrogen receptors (Tremblay *et al.,* 1997 and references therein), a logical approach to the treatment of estrogen-sensitive breast cancer is the use of anti-estrogens, or compounds that block the interaction of estrogens with their specific receptor. Unfortunately, until very recently, no agent with pure antiestrogenic activity under *in vivo* conditions has been available. Tamoxifen, the only anti-estrogen widely available for the treatment of women with breast cancer, has an efficacy comparable to that achieved with ablative and endocrine therapies (Powles, 1997). This limited clinical efficacy of tamoxifen may be related to its well-known mixed estrogenic and antiestrogenic activities, which are species-, tissue-, cell- and even gene-specific (Chalbos *et al.,* 1993; Gallo and Kaufman 1997; Jordan, 1994; Poulin *et al.,* 1989 and references therein). Furthermore, OH-tamoxifen cannot block the ligand-independent activation of the ER by growth factors and other factors stimulating the mitogen-activated protein (MAP) kinase (Kato *et al.,* 1995; Bunone *et al.,* 1996; Tremblay *et al.,* 1997).

The 7α-alkyl derivatives of estradiol (Wakeling, 1993) and estradiol derivatives possessing additional changes designed to increase their affinity for the estrogen receptor and to increase their bioavailability (Labrie *et al.,* 1992; Lévesque *et al.,* 1991) have been shown to possess pure and potent anti-estrogenic activity in most well-recognized *in vitro* and *in vivo* systems, including human breast cancer cells (Labrie *et al.,* 1992; Wakeling, 1993; Tremblay *et al.,* 1997). The 7α-alkyl estradiol derivative ICI 164384, however, has been found to possess some estrogenic agonistic activity in guinea pig uterine cells (Chetrite and Pasqualini, 1991; Giambiagi and Pasqualini, 1991). Furthermore, both OH-Tamoxifen and ICI 164384 can stimulate CAT activity in MCF-7 cells transfected with a *pS2-tkCAT* fusion gene (Weaver *et al.,* 1988). Moreover, such 7α-estradiol derivatives are difficult to synthesize, and their bioavailability by the oral route is limited, thus necessitating parenteral administration.

We have thus synthesized a series of benzopyrans, which are potent anti-estrogens in the most representative *in vitro* and *in vivo* model systems currently available and have high oral bioavailability. These compounds show a 1.5- to 2.9-times higher affinity than 17β-estradiol (E_2) for the estrogen receptor in human breast cancer and normal uterine cytosol and have no agonistic estrogenic activity in human breast cancer models studied *in vitro* and *in vivo* in nude mice (Couillard *et al.,* 1996; Gauthier *et al.,* 1997). The present study describes the effects of the novel non-steroidal antiestrogen EM-800 and related compounds on basal and E_2-induced cell proliferation in 3 well-characterized estrogen receptor-positive human breast cancer cell lines. The present results show that EM-800 and its active metabolite EM-652 are the most potent known anti-estrogens *in vitro* in human breast cancer cells and are, most importantly, devoid of intrinsic estrogenic activity.

MATERIAL AND METHODS

Chemicals

E_2 was obtained from Steraloids (Wilton, NH). All media and supplements for cell culture were from Sigma (St. Louis, MO), except fetal bovine serum (FBS), which was from Hyclone (Logan, UT). EM-343 and its (−) enantiomer EM-651, as well as its (+) active enantiomer EM-652, were synthesized in our laboratory. EM-762, its (−) enantiomer, EM-776, its (+) enantiomer, EM-800, the antiestrogen ICI 164384, ICI 182780, (Z)-4-OH-toremifene and droloxifene were also synthesized in our laboratory. (Z)-4-OH-TAM was kindly provided by Dr. D. Salin-Drouin, Besins-

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Iscovesco (Paris, France) while TAM (citrate salt) was from Sigma and toremifene (citrate salt) was kindly provided by Orion Farmos (Tucku, Finland). The structures of these antiestrogens and related compounds are illustrated in Figure 1.

Maintenance of stock cell cultures

ZR-75-1 human breast cancer cells were obtained from the ATCC (Rockville, MD) at passage 84 and were routinely grown in phenol red-free RPMI-1640 medium supplemented with 1 nM E_2, 2 mM L-glutamine, 1 mM sodium pyruvate, 15 mM HEPES, 100 IU penicillin/ml, 50 μg streptomycin sulfate/ml and 10% (v/v) FBS, as previously described (Poulin *et al.,* 1989; Simard *et al.,* 1990). This cell line was derived from a malignant ascitic effusion in a 63-year-old Caucasian female with infiltrating ductal carcinoma. ZR-75-1 cells were used between passages 90 and 98 and subcultured once a week. The T-47D human breast cancer cells were obtained from the ATCC (HTB 133) at passage 86 and routinely grown in the same medium described above for ZR-75-1 cells. The T-47D cell line was derived from a pleural effusion obtained from a 54-year-old female patient with infiltrating ductal carcinoma. T-47D cells were used between passages 90 and 120 and subcultured weekly.

MCF-7 human breast cancer cells were obtained from the ATCC (HTB 22) at passage 147 and routinely grown in phenol red-free Dulbecco's modified Eagle's-Ham's F12 medium, the supplements mentioned above and 5% FBS. The MCF-7 human breast adenocarcinoma cell line was derived from the pleural effusion of a 69-year-old female Caucasian patient. MCF-7 cells were used between passages 148 and 165 and subcultured weekly, as previously described (Simard *et al.,* 1990). MDA-MB-231 (ER-) human breast cancer cells were obtained from the ATCC (HTB 26) at passage 27 and routinely grown in phenol red-free minimal Eagle's-medium, the supplements mentioned above, 5% FBS and MEM non-essential amino acids (GIBCO BRL, Gaithersburg, MD). The MDA-MB-231 human breast adenocarcinoma cell line was derived from the pleural effusion of a 51-year-old female Caucasian patient. MDA-MB-231 cells were used between passages 27 and 40 and subcultured weekly. All cell lines were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

Cell proliferation studies

Cells in their late logarithmic growth phase were harvested with 0.1% pancreatin (Sigma) and resuspended in the appropriate medium containing 50 ng bovine insulin/ml and 5% (v/v) FBS treated twice with dextran-coated charcoal to remove endogenous steroids. Cells were plated in 24-well Falcon plastic culture plates (2 cm^2/well) at the indicated density and allowed to adhere to the surface of the plates for 72 hr. Thereafter, medium was replaced with fresh medium containing the indicated concentrations of compounds diluted from 1,000 × stock solutions in 99% redistilled ethanol in the presence or absence of E_2. Control cells received only the ethanolic vehicle (0.1% EtOH, v/v). Cells were incubated for the specified time intervals with medium changes at 2- or 3-day intervals. Cell number was determined by measurement of DNA content as previously described (Simard *et al.,* 1990).

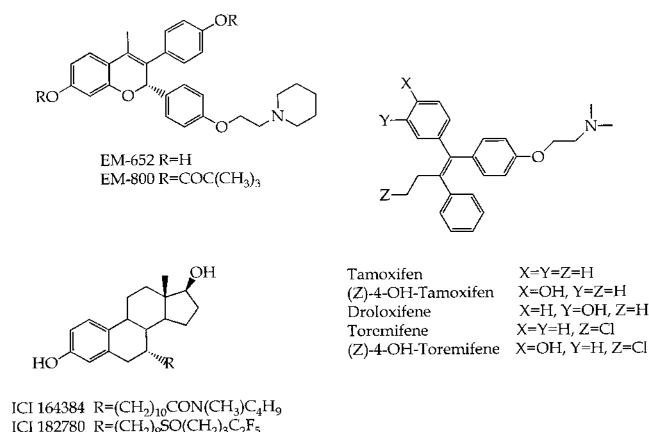

FIGURE 1 – Chemical structures of antiestrogens.

Cell kinetic parameters

MCF-7 cells were plated in 6-well Falcon plastic plates at an initial density of 1×10^5 cells/well and allowed to adhere for 72 hr. The cells were then pretreated for 3 days with the indicated concentrations of compounds in the presence or absence of 0.1 nM E_2 before changing for fresh medium containing the same compounds and 10 μM BrdUrd. After a 2-day treatment in the presence of BrdUrd, cells were harvested and briefly centrifuged (400*g*). After resuspension in 0.3 ml PBS, the cells were fixed by addition of 0.7 ml of 95% ethanol. Fixed cells were centrifuged, washed with PBS (pH 7.2) and resuspended in PBS containing 1 mg RNase (40 U/ml)/10^6 cells for 30 min at 37°C. Cells were then separated by centrifugation before resuspension in 0.5 ml of staining buffer consisting of 100 mM Tris, 154 mM NaCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.1% (v/v) and 1.2 μg/ml Hoechst (Frankfurt, Germany) 33258, pH 7.4. After 15 min on ice, propidium iodide (PI) was added from a stock solution to give a final concentration of 2 μg/ml.

The cells were then analyzed with a Coulter (Hialeah, FL) EPICS 753 pulse cytometer with 2 Argon lasers as described (Ormerod and Kubbies, 1992). The percentage of cells that had incorporated BrdUrd during the S phase (BrdUrd-positive) was determined after UV excitation at 350 nm (40 mW) and emission between 408 and 505 nm. The distribution of cells throughout the phases of the cell cycle was evaluated by measuring the red fluorescence (>610 nm) emitted by PI after excitation of the cells at 488 nm (400 mW). Debris and doublets were eliminated by plotting integrated red fluorescence *vs.* peak red fluorescence and setting a bip map around the events on the diagonal. A bivariate cytogram of blue (408–505 nm) *vs.* red (>610 nm) was also recorded. In this cytogram, the BrdUrd-positive cells show "quenching" with the Hoechst dye and were found outside of the diagonal zone. The double staining showed the relative proportion of BrdUrd-positive cells in the cell cycle.

Calculations and statistical analysis

Dose-response curves as well as EC_{50} and IC_{50} values were calculated using a weighted iterative non-linear least-squares regression (Rodbard, 1974). All results are expressed as means ± SEM, except when SEM overlaps with the symbol used, in which instance only the symbol is illustrated. The apparent inhibition Ki values were calculated according to the following equation: $Ki = IC_{50}/(1 + S/K)$ (Cheng and Prusoff, 1973). In this equation, S represents the concentration of E_2, K is the apparent K_D of E_2 action on cell proliferation [*i.e.,* ZR-75-1 cells (0.0198 nM); T-47D cells (0.0112 nM); MCF-7 cells (0.006 nM)] and IC_{50} is the concentration of the antiestrogen giving a 50% inhibition of E_2 action on cell growth.

RESULTS

Comparison of the effects of EM-800 and OH-TAM on basal and E_2-induced cell proliferation in T-47D, ZR-75-1 and MCF-7 human breast cancer cell lines

We first investigated the effect of increasing concentrations of EM-800, its (−) enantiomer, EM-776, and EM-762, the mixture of these 2 enantiomers, compared with the effect of OH-TAM on basal and E_2-induced cell proliferation in T-47D (Fig. 2), ZR-75-1 (Fig. 3) and MCF-7 (Fig. 4) cells. A 9-day incubation period was selected to obtain an optimal amplitude of the mitogenic effect of E_2 (Simard *et al.,* 1990). As illustrated in Figure 2, a 9-day exposure to 0.1 nM E_2 increased the proliferation of T-47D cells by 3.22-fold. This E_2-induced stimulation of cell proliferation was

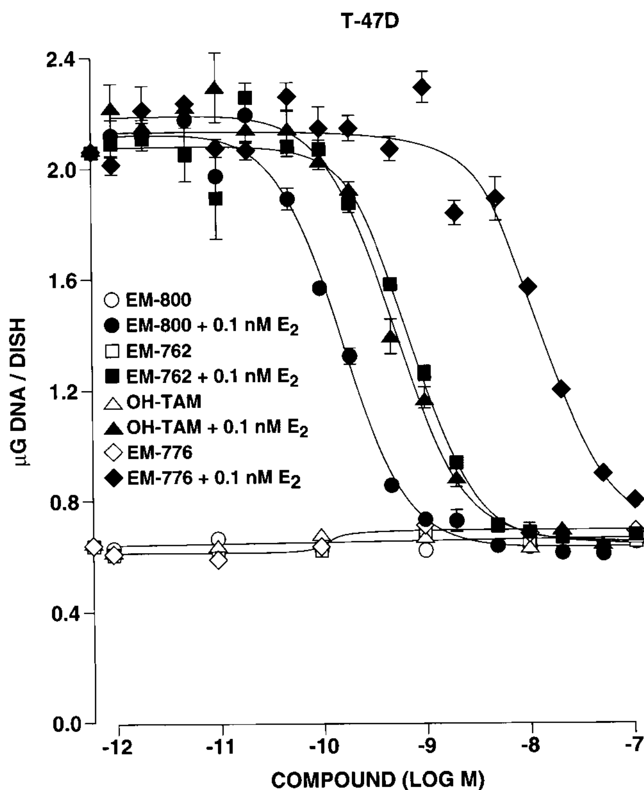

FIGURE 2 – Effect of increasing concentrations of EM-800, its (−) enantiomer EM-776, EM-762 (mixture of the 2 enantiomers) or 4-OH-*trans*-tamoxifen (OH-TAM) on basal and E_2-induced cell proliferation in T-47D human breast cancer cells. Three days after plating at an initial density of 7.5×10^3/2-cm² well, cells were exposed for 9 days to the indicated concentrations of compounds in the presence or absence of 0.1 nM E_2. Media were changed at 2- or 3-day intervals. At the end of the incubation period, cell number was determined by measurement of DNA content. Data obtained with control medium alone in the presence or absence of 0.1 nM E_2 are indicated on the Y axis. The data are expressed as the means ± SEM of triplicate dishes. When the SEM overlaps with the symbol used, only the symbol is illustrated. The apparent inhibition Ki values of EM-800, EM-762, OH-TAM and EM-776 for their effect on E_2-induced cell proliferation were calculated at 0.017, 0.079, 0.054 and 1.33 nM, respectively. The Ki values were calculated as described in Material and Methods.

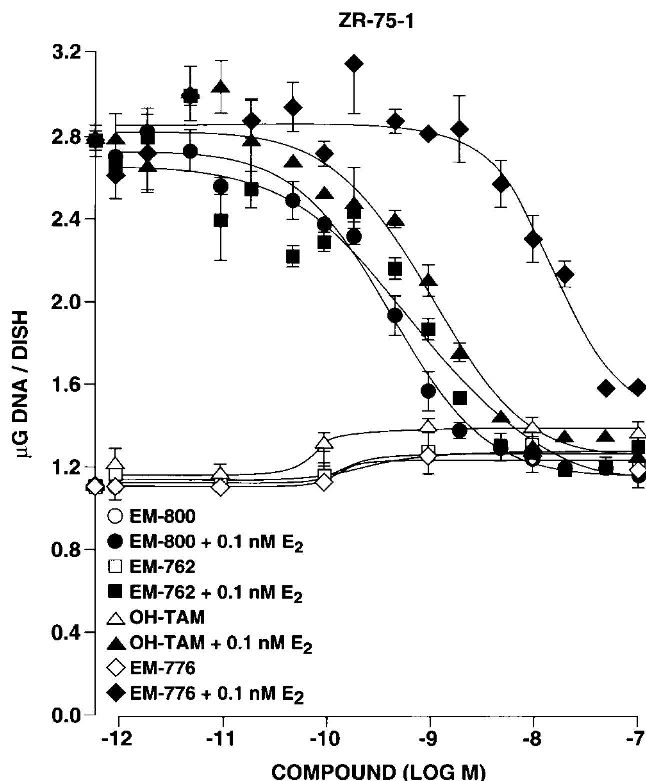

FIGURE 3 – Effect of increasing concentrations of EM-800, its (−) enantiomer EM-776, EM-762 (mixture of the 2 enantiomers) or 4-OH-*trans*-tamoxifen (OH-TAM) on basal and E_2-induced cell proliferation in ZR-75-1 human breast cancer cells. Three days after plating at an initial density of 1.2×10^4/2-cm² well, cells were exposed for 10 days to the indicated concentrations of compounds in the presence or absence of 0.1 nM E_2. Media were changed at 2- or 3-day intervals. Data are expressed as described in the legend of Figure 2. The apparent inhibition Ki values of EM-800, EM-762, OH-TAM and EM-776 for their effect on E_2-induced cell proliferation were calculated at 0.070, 0.126, 0.165 and 2.62 nM, respectively.

competitvely blocked by simultaneous incubation with EM-800, EM-762, OH-TAM or EM-776 at respective IC_{50} values of 0.165, 0.786, 0.536 and 13.18 nM (Fig. 2). It can also be seen in Figure 2 that these compounds did not affect basal cell proliferation when incubated alone, thus indicating the absence of intrinsic agonistic estrogenic activity.

As illustrated in Figure 3, after a 10-day incubation with increasing concentrations of EM-800, EM-762, OH-TAM or EM-776, the 2.46-fold increase in ZR-75-1 cell proliferation induced by 0.1 nM E_2 was competitively reversed at respective IC_{50} values of 0.422, 0.763, 1.00 and 15.84 nM. Furthermore, after a 10-day incubation with increasing concentrations of EM-762, EM-776 or EM-800 in the absence of E_2, basal ZR-75-1 cell proliferation was not significantly affected while OH-TAM, at concentrations of 0.1–100 nM, caused a 15–20% increase in cell proliferation ($p < 0.01$).

The 2.83-fold increase in MCF-7 cell proliferation induced by a 9-day incubation with 0.1 nM E_2 was competitively blocked by simultaneous exposure to EM-800, EM-762, OH-TAM or EM-776 at respective IC_{50} values of 0.581, 1.038, 1.782 and approx. 50 nM (Fig. 4). When added alone, OH-TAM at the concentrations of 0.01–1 nM caused a 10–17% ($p < 0.05$) stimulation of MCF-7 cell proliferation in the absence of E_2. EM-776 caused an isolated 15% stimulation of basal cell proliferation at 0.1 and 1 nM ($p < 0.05$).

Comparison of the effects of EM-652 and EM-800 with those of ICI 164384, OH-TAM and TAM on basal and E_2-induced cell proliferation in T-47D, ZR-75-1 and MCF-7 human breast cancer cell lines

Since EM-800 is rapidly metabolized into the active compound EM-652 in intact cells, we next compared the effect of increasing concentrations of the non-steroidal antiestrogen EM-652 and EM-800 with those of OH-TAM and TAM and of the steroidal antiestrogen ICI 164384 on basal and E_2-induced cell proliferation in T-47D (Fig. 5), ZR-75-1 (Fig. 6) and MCF-7 (Fig. 7) cells. As illustrated in Figure 5, a 10-day exposure to 0.1 nM E_2 increased the proliferation of T-47D cells by 4.77-fold. This E_2-induced stimulation of cell proliferation was competitively blocked by simultaneous incubation with EM-800, EM-652, OH-TAM, ICI 164384 and TAM at respective IC_{50} values of 0.148, 0.146, 0.522, 2.41 and approx. 100 nM (Fig. 5). It can also be seen in Figure 5 that these compounds did not affect basal T-47D cell proliferation when incubated alone.

As illustrated in Figure 6, after a 9-day incubation of ZR-75-1 cells with increasing concentrations of EM-800, EM-652, OH-TAM, ICI 164384 or TAM, the 2.01-fold increase in cell proliferation induced by 0.1 nM E_2 was competitively reversed at respective

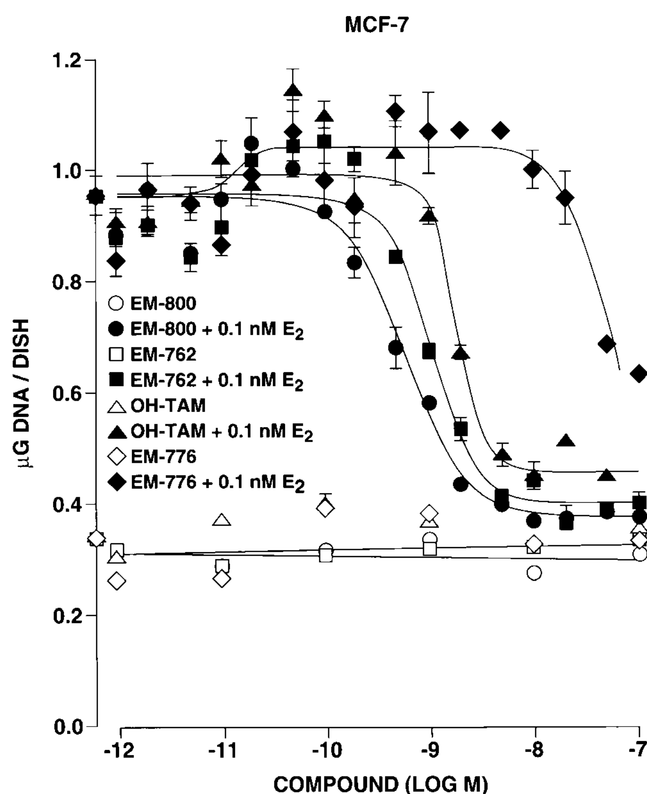

FIGURE 4 – Effect of increasing concentrations of EM-800, its (−) enantiomers EM-776, EM-762 (mixture of the 2 enantiomers) or 4-OH-*trans*-tamoxifen (OH-TAM) on basal and E_2-induced cell proliferation in MCF-7 human breast cancer cells. Three days after plating at an initial density of 0.8×10^4/2-cm^2 well, cells were exposed for 9 days to the indicated concentrations of compounds in the presence or absence of 0.1 nM E_2. Media were changed at 2- or 3-day intervals. Data are expressed as described in the legend of Figure 2. The Ki values of EM-800, EM-762, OH-TAM and EM-776 for their effect on E_2-induced cell proliferation were calculated at 0.033, 0.059, 0.101 and approx. 2.83 nM, respectively.

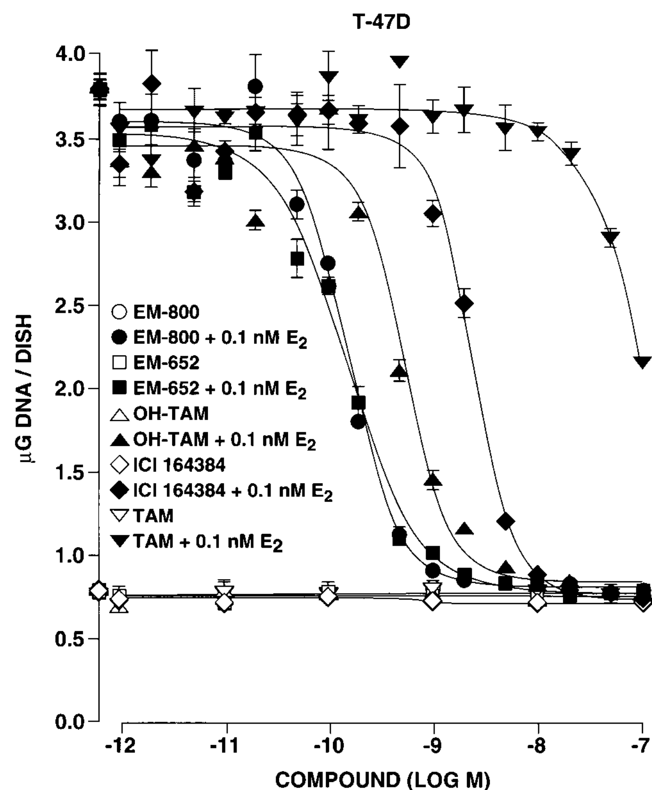

FIGURE 5 – Effect of increasing concentrations of EM-652, EM-800, ICI 164384, 4-OH-*trans*-tamoxifen (OH-TAM) or tamoxifen (TAM) on basal and E_2-induced cell proliferation in T-47D human breast cancer cells. Three days after plating, at an initial density of 7.5×10^3/2-cm^2 well, cells were exposed for 10 days to the indicated concentrations of compounds in the presence or absence of 0.1 nM E_2. Media were changed at 2- or 3-day intervals. Data are expressed as described in the legend of Figure 2. The Ki values of EM-800, EM-652, OH-TAM, ICI 164384 and TAM for their effect on E_2-induced cell proliferation were calculated at 0.015, 0.015, 0.053, 0.243 and approx. 10 nM, respectively.

IC_{50} values of 0.475, 0.750, 0.646, 4.88 and approx. 100 nM. Furthermore, basal cell proliferation was not significantly affected after a 9-day incubation with increasing concentrations of EM-652 or EM-800 in the absence of E_2. In the absence of estrogens, OH-TAM, on the other hand, led to a 10% ($p < 0.05$) stimulation of cell proliferation.

The 8.43-fold increase in MCF-7 cell proliferation induced by a 9-day incubation with 0.1 nM E_2 was competitively blocked by a simulateneous exposure to EM-800, EM-652, OH-TAM or ICI 164384 at respective IC_{50} values of 0.582, 0.321, 1.06 and 3.49 nM (Fig. 7). However, such an incubation with 50 and 100 nM TAM was only able to reverse by approx. 20% the E_2-induced MCF-7 cell proliferation. It can also be seen in Figure 7 that after a 9-day incubation with increasing concentrations of EM-652 or EM-800 in the absence of E_2, the basal proliferation of MCF-7 cells was not affected significantly. OH-TAM, on the other hand, at concentrations of 0.1–10 nM, caused 35–55% ($p < 0.01$) stimulation of cell proliferation in the absence of E_2.

Comparison of the effect of EM-800 with those of droloxifene, OH-toremifene, toremifene and ICI 182780 on basal and E_2-induced human breast cancer cell proliferation in T-47D and MCF-7 human breast cancer cells

Since droloxifene, toremifene and ICI 182780 are being developed for the treatment of breast cancer (Löser *et al.,* 1985; Valavaara, 1990; Wakeling and Bowler, 1992), it was of interest to compare the effect of these 3 compounds on breast cancer cell proliferation with that of EM-800. As illustrated in Figure 8, a 9-day exposure to 0.1 nM E_2 increased by 3.8-fold the proliferation of T-47D cells. This E_2-induced stimulation of cell proliferation was competitively blocked by simultaneous incubation with EM-800, OH-TAM, ICI 182780, droloxifene or toremifene at respective IC_{50} values of 0.158, 0.400, 0.434, 7.30 and $>$100 nM (Fig. 8).

After 8 days of treatment of MCF-7 cells in the absence of E_2, OH-TAM, droloxifene, and toremifene all led to a 75–100% increase in cell proliferation that was dose dependent: the stimulation by OH-TAM was already observed at 0.01 nM, the lowest concentration used, while droloxifene gave the first significant effect at 1 nM and toremifene stimulated basal MCF-7 cell proliferation at 100 nM (Fig. 9). In the same experiment, EM-652, OH-TAM, EM-651 (the "inactive" enantiomer of EM-652), droloxifene and toremifene inhibited the proliferative action of E_2 at respective IC_{50} values of 0.193, 0.731, 22.28, 30.58 and $>$500 nM.

As illustrated in Figure 10, after a 9-day incubation with increasing concentrations of EM-800, OH-toremifene or toremifene, the 2.47-fold increase in cell proliferation induced by 0.1 nM E_2 was completely reversed at respective IC_{50} values of 0.112, 0.430 and 179 nM.

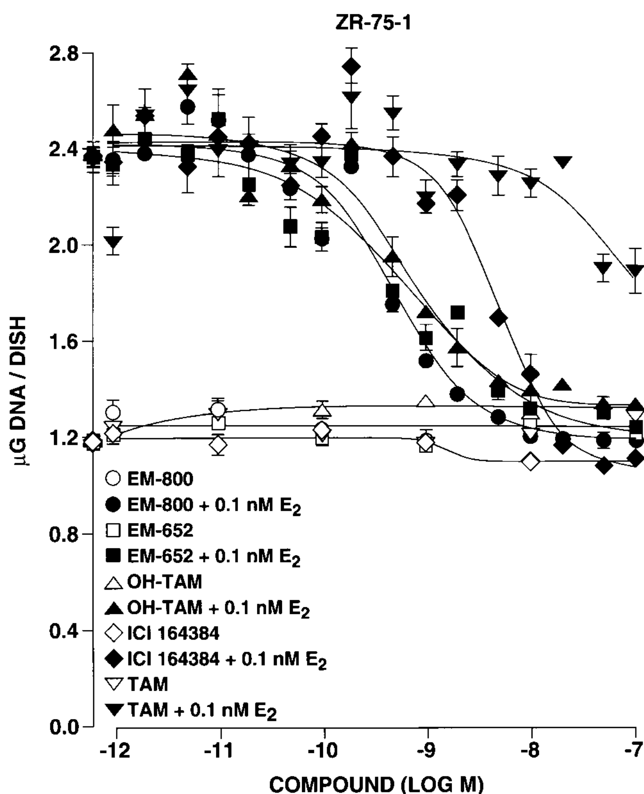

FIGURE 6 – Effect of increasing concentrations of EM-652, EM-800, ICI 164384, 4-OH-*trans*-tamoxifen (OH-TAM) or tamoxifen (TAM) on basal and E_2-induced cell proliferation in ZR-75-1 human breast cancer cells. Three days after plating at an initial density of 1.1 × 10^4/2-cm^2 well, cells were exposed for 9 days to the indicated concentrations of compounds in the presence or absence of 0.1 nM E_2. Media were changed at 2- or 3-day intervals. Data are expressed as described in the legend of Figure 2. The Ki values of EM-800, EM-652, OH-TAM, ICI 164384 and TAM for their effect on E_2-induced cell proliferation were calculated at 0.079, 0.124, 0.107, 0.807 and >16.5 nM, respectively.

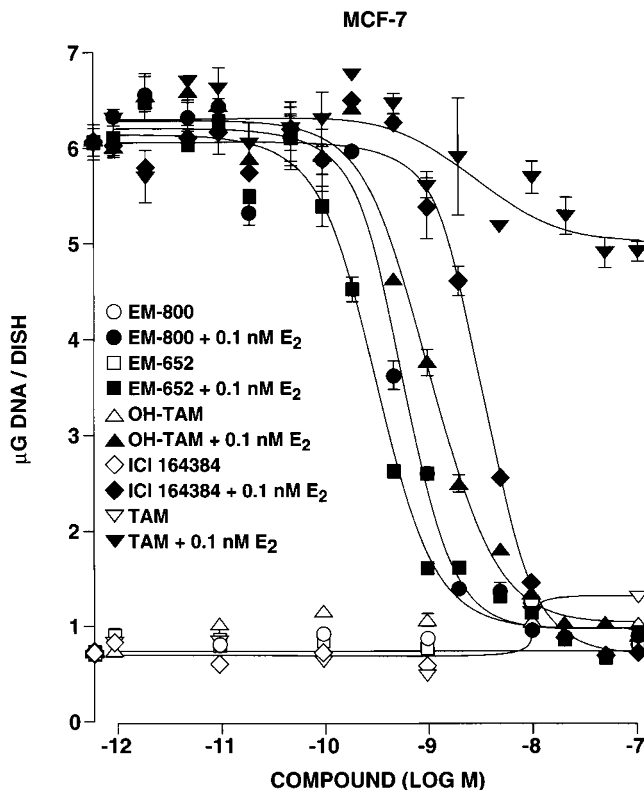

FIGURE 7 – Effect of increasing concentrations of EM-652, EM-800, ICI 164384, 4-OH-*trans*-tamoxifen (OH-TAM) or tamoxifen (TAM) on basal and E_2-induced cell proliferation in MCF-7 human breast cancer cells. Three days after plating, at an initial density of 1 × 10^4/2-cm^2 well, cells were exposed for 9 days to the indicated concentrations of compounds in the presence or absence of 0.1 nM E_2. Media were changed at 2- or 3-day intervals. Data are expressed as described in the legend of Figure 2. The Ki values of EM-800, EM-652, OH-TAM and ICI 164384 for their effect on E_2-induced cell proliferation were calculated at 0.033, 0.018, 0.060 and 0.197 nM, respectively.

Effect of EM-800, EM-343, EM-776 and ICI 164384 on cell proliferation of the MDA-MB-231 human breast cancer cell line

We then studied the effect of increasing concentrations of EM-800, EM-343, EM-776 and ICI 164384 after a 9-day incubation in the presence or absence of 0.1 nM E_2 in the estrogen-insensitive MDA-MB-231 human breast cancer cell line. As illustrated in Figure 11, EM-800, EM-343 and EM-776 failed to change the proliferation of MDA-MB-231 cells significantly in the presence or absence of E_2, whereas the high concentration of ICI 164384 had a non-significant tendency to decrease the value of this parameter.

Comparison of the effect of EM-652, EM-800, OH-TAM and TAM on the proportion of cycling MCF-7 cells

To assess the percentage of MCF-7 cells that progressed through the S-phase of the cycle during incubation with EM-652, EM-800 or TAM in the presence or absence of E_2, the continous BrdUrd exposure technique was used. As measured after a 48-hr exposure to BrdUrd, 72-hr pretreatment with 1 nM EM-652, EM-800 or OH-TAM alone decreased the percentage of BrdUrd-positive cells from 43.6% to 20.2%, 21.5% and 30.9%, respectively ($p < 0.01$) (Fig. 12*a*). On the other hand, incubation with 0.1 nM E_2 increased the percentage of BrdUrd-positive cells to 77.9% ($p < 0.01$). Addition of increasing concentrations of EM-652, EM-800 or OH-TAM completely blocked the stimulatory effect of E_2 on this parameter and caused a further decrease below the control value to reach levels similar to those obtained with these compounds in the absence of E_2 (Fig. 12*b*). The inhibitory effect of EM-652, EM-800 and OH-TAM on the percentage of BrdUrd-positive cells was observed at respective IC_{50} values of 0.60, 1.26 and 3.8 nM. It can also be seen in Figure 12 that treatment with TAM was approximately 1,000-fold less effective in decreasing the proportion of cycling MCF-7 cells.

DISCUSSION

Our present data show that the novel non-steroidal compound EM-800 and its metabolite EM-652 exert the most potent antagonistic effects of the compounds tested on E_2-induced proliferation in T-47D, ZR-75-1 and MCF-7 human breast cancer cells in culture. Furthermore, our data indicate that these 2 antiestrogens are devoid of intrinsic estrogenic activity, as evidenced by the absence of stimulatory effect on basal cell proliferation in 3 estrogen-sensitive human breast cancer cell lines. Our observations thus strongly suggest that EM-652 and EM-800 are pure antiestrogens devoid of partial agonist activity in human breast cancer tissue.

It is also of interest to observe that the antiestrogenic activity of EM-652 and EM-800 on E_2-induced cell proliferation in T-47D cells is at least 2 orders of magnitude more potent than TAM, 2.5-

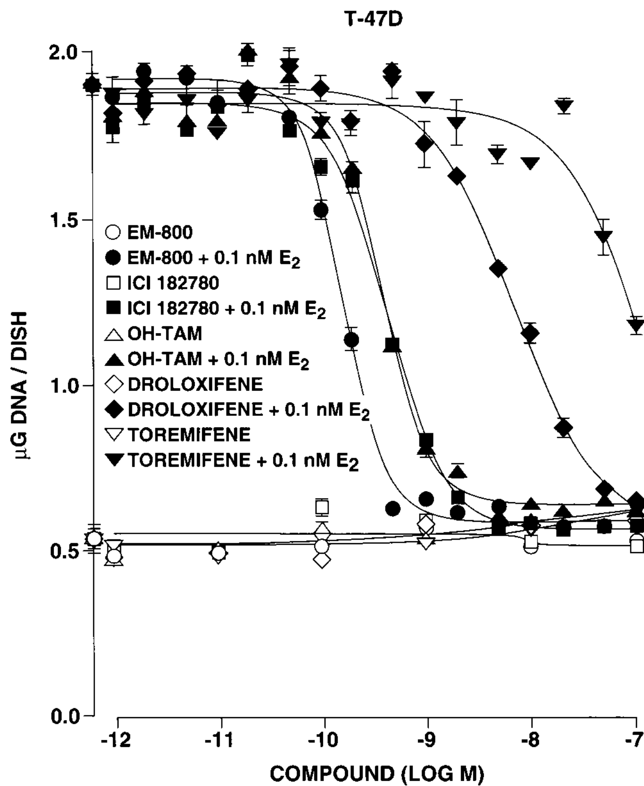

FIGURE 8 – Effect of increasing concentrations of EM-800, ICI 182780 or 4-OH-*trans*-tamoxifen (OH-TAM) droloxifene or toremifene on basal and E_2-induced cell proliferation in T-47D human breast cancer cells. Three days after plating, cells were exposed for 9 days to the indicated concentrations of compounds in the presence or absence of 0.1 nM E_2. Media were changed at 2- or 3-day intervals. Data are expressed as described in the legend of Figure 2. The Ki values of EM-800, OH-TAM, ICI 182780, droloxifene or toremifene for their effect on E_2-induced cell proliferation were calculated at 0.016, 0.040, 0.044, 0.735 and >10 nM, respectively.

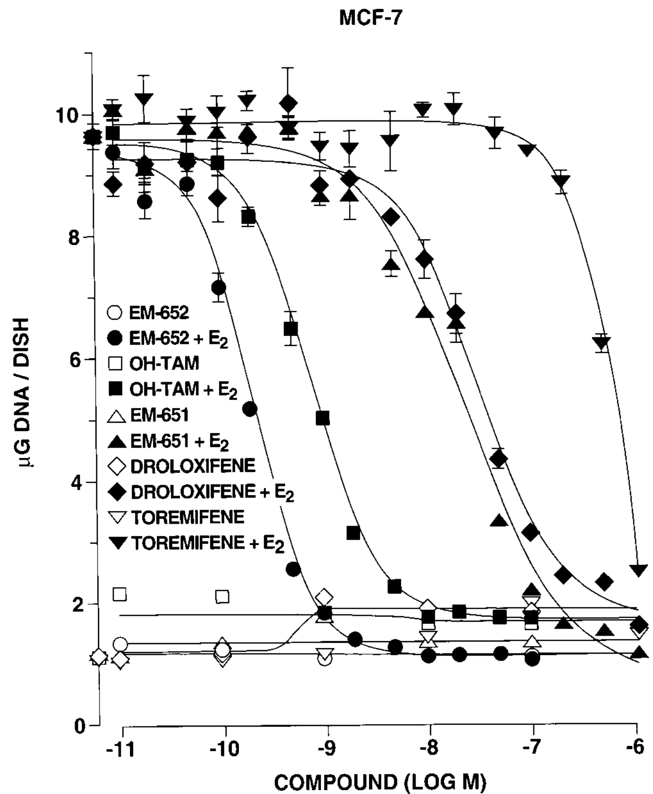

FIGURE 9 – Effect of increasing concentrations of EM-652, 4-OH-*trans*-tamoxifen (OH-TAM), EM-651, droloxifene or toremifene on basal and E_2-induced cell proliferation in MCF-7 human breast cancer cells. Three days after plating, at an initial density of 1×10^4/2-cm^2 well, cells were exposed for 8 days to the indicated concentrations of compounds in the presence or absence of 0.1 nM E_2. Media were changed at 2- or 3-day intervals. Data are expressed as described in the legend of Figure 2. The Ki values of EM-652, OH-TAM, EM-651, droloxifene, and toremifene for their effect on E_2-induced cell proliferation were calculated at 0.011, 0.041, 1.29, 1.73, and >28 nM.

to 3.6-fold more potent than OH-TAM, and 3.84-, 2.74- and 16.3-fold more potent than OH-toremifene, ICI 182780 and ICI 164384, respectively. EM-800 is 46- and 1,600-fold more potent than droloxifene and toremifene in inhibiting E_2-induced T-47D cell proliferation.

Our results also show that EM-652 and EM-800 decrease the proportion of MCF-7 cells that advanced through the S-phase and completely blocked the stimulatory effect of E_2 on this parameter. In fact, EM-652 and EM-800 were at least 1,000-fold more effective than TAM in reducing the proportion of BrdUrd-positive cells in the presence or absence of E_2 while EM-652 was 6-fold more potent than OH-TAM in the presence of E_2.

In the present study, we also demonstrated that the (−) enantiomers EM-651 and 776 have low activity in inhibiting E_2-induced cell proliferation in breast cancer cell lines compared with their respective (+) enantiomers, EM-652 and EM-800. The antiestrogenic activity of these (−) enantiomers has been estimated to be approximately 1% that of the (+) enantiomers in T-47D cells. It is likely that the antiestrogenic effect displayed by the (−) enantiomer EM-651 relative to EM-652 corresponds to true activity since this enantiomer was 99.95% optically pure.

Our data are in agreement with the much higher affinity of OH-TAM than TAM for the estrogen receptor (Robertson *et al.,* 1982). The extremely low activity of TAM in the 3 human breast cancer cell lines studied indicates that 4-hydroxylation of TAM does not occur at a significant rate in these cells and that hydroxylation of TAM must take place in other tissues. Since EM-800 has a 125-times lower affinity for the estrogen receptor in human breast cancer tissue than EM-652 (Gauthier *et al.,* 1997), the almost equipotent activity of EM-800 and its metabolite EM-652 observed in the present study suggests that breast cancer cells quantitatively transform EM-800 into EM-652, thus making optimal use of the compound. Although such predictions remain to be confirmed by pharmacokinetic studies in women, a comparable difference in the potencies of EM-800 and TAM has been observed *in vivo* in mice on uterine weight where EM-800 has been estimated to be much more potent than TAM and to cause a much greater maximal inhibitory effect than TAM (Gauthier *et al.,* 1997). Moreover, EM-800 administered by oral route is 2- to 3-fold more potent than ICI 182780 administered subcutaneously on estrogen-sensitive parameters in ovariectomized mice (Martel *et al.,* 1996).

OH-TAM is present at only 1.4% the concentration of TAM in breast cancer tissue in women receiving TAM chronically. The situation is similar in the serum, where the concentration of OH-TAM is 2.2% that of TAM (Lien *et al.,* 1991). Since EM-800 is almost completely transformed into the active metabolite EM-652, while only approximately 2% of TAM is converted into the active metabolite OH-TAM, it is reasonable to consider that 98% of administered and absorbed TAM does not act as antiestrogen in the organism. In other words, close to 100% of absorbed EM-800 should play an antiestrogenic action in breast cancer cells, while only 2% of TAM plays a useful role, thus leaving 98% of

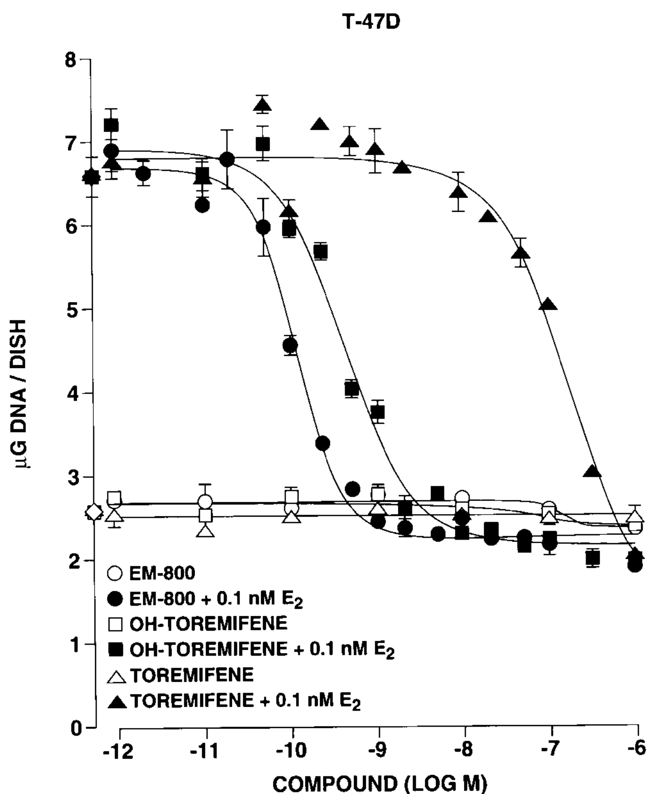

FIGURE 10 – Effect of increasing concentrations of EM-800, 4-OH-*trans*-toremifene (OH-toremifene) or toremifene on basal and E_2-induced cell proliferation in T-47D human breast cancer cells. Three days after plating, cells were exposed for 9 days to the indicated concentrations of compounds in the presence or absence of 0.1 nM E_2. Media were changed at 2- or 3-day intervals. Data are expressed as described in the legend of Figure 2. The Ki values of EM-800, OH-toremifene or toremifene for their effect on E_2-induced cell proliferation were calculated at 0.011, 0.043 and 18 nM, respectively.

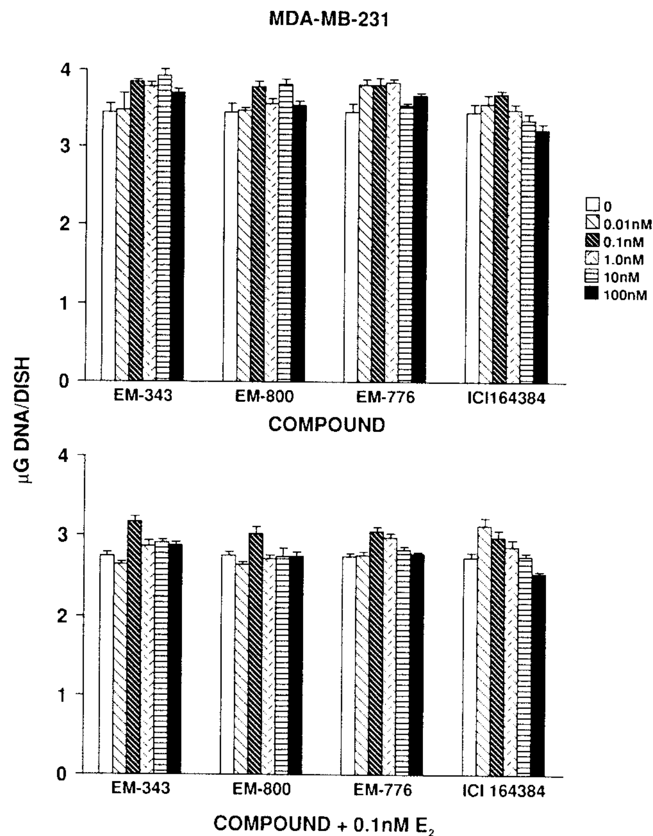

FIGURE 11 – Effect of increasing concentrations of EM-800, EM-343, EM-776, or ICI 164384 on the proliferation of MDA-MB231 (ER-) human breast cancer cells. Two days after plating at an initial density of 8.5 × 10^3/2-cm^2 well, cells were exposed for 9 days to the indicated concentrations of compounds in the presence or absence of 0.1 nM E_2. Media were changed at 2- or 3-day intervals. Data are expressed as described in the legend of Figure 2.

absorbed and circulating TAM for potential side effects not compensated for by benefits related to blockade of the estrogen receptor.

In view of the high potency of these 2 novel antiestrogens and their pure antiestrogenic characteristics as measured on the proliferation of estrogen-sensitive human breast cancer cells, it can be anticipated that these antiestrogens should achieve a more complete blockade of the action of estrogens on tumor growth, thus resulting in more rapid, more complete and longer-lasting responses compared with TAM.

Most importantly, EM-800 and EM-652 have no estrogenic activity in the 3 breast cancer cell lines studied while OH-TAM and droloxifene cause a significant stimulation of ZR-75-1 and or MCF-7 human breast cancer cell proliferation. The estrogenic activity of TAM is illustrated by studies demonstrating tumor response to withdrawal of TAM at time of treatment failure (Canney *et al.,* 1987; Howell *et al.,* 1992). Such data suggest that progression of the disease observed in patients treated with TAM is related to the stimulation of the cancer by the estrogenic activity of TAM. These clinical data are well supported by the stimulation induced by TAM of breast cancer cells cultured directly from patients (DeFriend *et al.,* 1994 and references therein). The observations of a stimulatory effect of TAM on breast cancer cell growth are in agreement with the stimulatory effect of TAM or OH-TAM observed on the growth of breast cancer cells demonstrated repeatedly *in vitro* (DeFriend *et al.,* 1994; Poulin *et al.,* 1989; Weaver *et al.,* 1988 and references therein) as well as *in vivo* (Gottardis and Jordan, 1988).

EM-800 and EM-652 have been shown to be devoid of estrogenic activity in human Ishikawa uterine cancer cells, as suggested by the absence of a stimulatory effect on the alkaline phosphatase activity (Simard *et al.,* 1997). Moreover, in contrast to EM-800 and EM-652, OH-TAM, 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 EM-800 or EM-652 (Gauthier *et al.,* 1997; Simard *et al.*, 1997). These findings thus suggest that these novel antiestrogens should avoid the increased risk of uterine carcinoma associated with long-term administration of Tamoxifen in women (Jordan and Assikis 1995; MacMahon, 1997).

Clinical trials are under way to assess safety and tolerance and to obtain information on the efficacy of EM-800 in patients in progression after treatment with Tamoxifen following a positive response to Tamoxifen.

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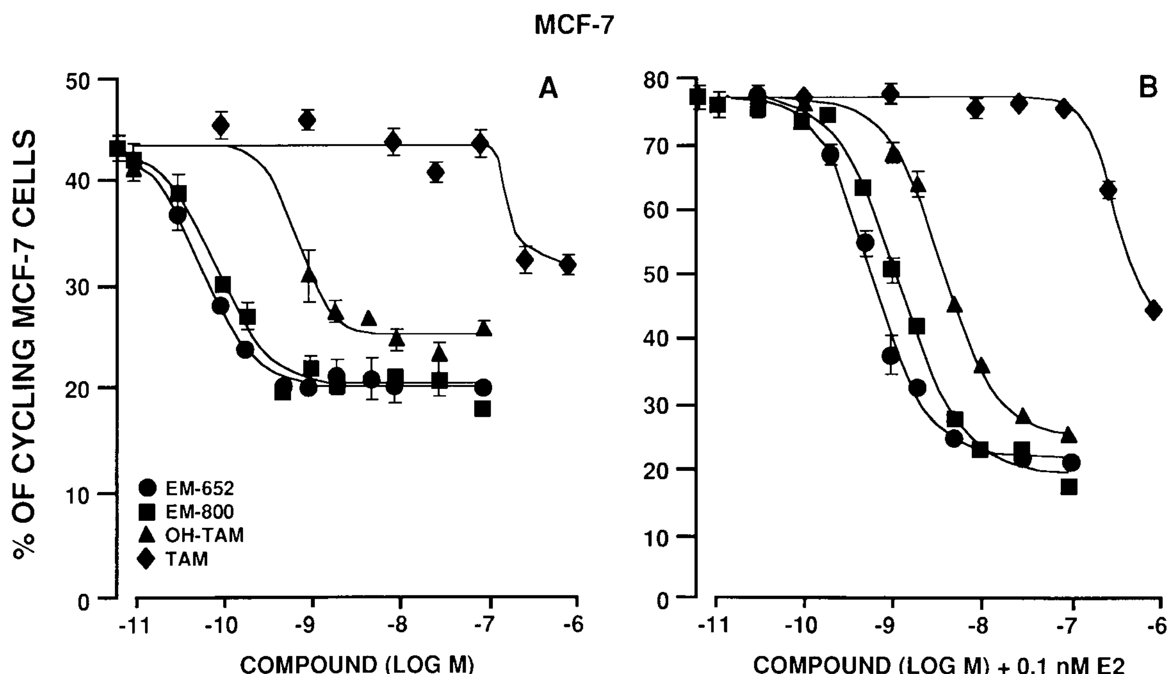

FIGURE 12 – Effect of increasing concentrations of EM-652, EM-800, OH-Tamoxifen (OH-TAM) or Tamoxifen (TAM) on the proportion of cycling MCF-7 cells after exposure to BrdUrd. Three days after plating at an initial density of 0.85×10^5/10-cm² well, cells were pretreated for 3 days with the indicated concentrations of compounds in the presence (*b*) or absence (*a*) of 0.1 nM E_2 before changing to fresh medium containing the same compounds and 10 μM BrdUrd. Cells were then harvested after 2 days, fixed and stained with the dye Hoechst 33358. The percentage of BrdUrd-positive cells was calculated as described in Material and Methods. Data obtained with control medium alone in the presence or absence of 0.1 nM E_2 are indicated on the Y axis. The data are expressed as the means ± SEM of triplicate dishes. When the SEM overlaps with the symbol used, only the symbol is illustrated.

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