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Concentration-dependent mitogenic and antiproliferative actions of 2-methoxyestradiol in estrogen receptor-positive human breast cancer cells

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Abstract

We compared in this study the effects of 2-methoxyestradiol (2-MeO-E_2) on the growth of two estrogen receptor (ER)-negative human breast cancer cell lines (MDA-MB-231 and MDA-MB-435s) and two ER-positive human breast cancer cell lines (MCF-7 and T-47D). 2-MeO-E_2 exerted a concentration-dependent antiproliferative action in the ER-negative MDA-MB-231 and MDA-MB-435s cells. The presence or absence of exogenous 17β-estradiol (E_2) in the culture medium did not affect the potency and efficacy of 2-MeO-E_2's antiproliferative action in these ER-negative cells. When the ER-positive MCF-7 and T-47D cells were cultured in a medium supplemented with 10 nM of exogenous E_2, 2-MeO-E_2 at 750 nM to 2 μM concentrations exerted a similar antiproliferative effect. However, when the ER-positive cell lines were cultured in the absence of exogenous E_2, 2-MeO-E_2 at relatively low concentrations (10–750 nM) had a moderate mitogenic effect, with its apparent efficacy 75–80% of that of E_2. This mitogenic effect of 2-MeO-E_2 was ER-mediated and largely attributable to 2-MeO-E_2's residual estrogenic activity on the basis of our following findings: (i) its effect was only manifested in the ER-positive cells but not in the ER-negative cells; (ii) its effect in the ER-positive cells was partially or fully abolished when exogenous E_2 was concomitantly present in the culture medium; (iii) 2-MeO-E_2 retained 1–2% of E_2's binding affinity for the human ERα and ERβ, and its mitogenic effect was inhibited in a concentration-dependent manner by ICI-182,780, a pure ER antagonist; and (iv) its effect was not due to its metabolic conversion to 2-hydroxyestradiol. Our timely findings are of importance to the on-going clinical trials designed to evaluate 2-MeO-E_2's effectiveness for the treatment of different types (ER-positive or ER-negative) of human breast cancer. This knowledge will improve the design of clinical trials as well as the interpretation of clinical outcomes when 2-MeO-E_2 is used as a single agent therapy or as part of a combination therapy for human breast cancer.

Keywords: Bioactive estrogen metabolites; 2-Methoxyestradiol; Estrogen metabolism; Breast cancer; Chemotherapy

1. Introduction

2-Methoxyestradiol (2-MeO-E_2) is a nonpolar endogenous E_2 metabolite formed by the COMT-mediated *O*-methylation of 2-hydroxyestradiol, a major catechol E_2 metabolite formed in humans (reviewed in [1,2]). Several earlier studies have indicated that the endogenous monomethylated estrogen metabolites, including 2-MeO-E_2, have very weak binding affinity for the rat uterine ER and have little or no uterotropic activity in ovariectomized female rats [3–5]. This finding has led to the earlier suggestion that the COMT-mediated *O*-methylation of endogenous catechol estrogens primarily is a metabolic inactivation process (just like the *O*-methylation of endogenous catecholamines). In the past several years, however, studies have also shown that 2-MeO-E_2 at pharmacological concentrations has strong antiproliferative and apoptotic actions in a number of human cancer cell lines in culture [1,6–19]. Among many different types of cancer cells tested, several human breast cancer cell lines appeared to be highly sensitive to the strong antiproliferative actions of 2-MeO-E_2 in vitro [11,12]. Additional studies also showed that 2-MeO-E_2 at high doses inhibited the growth of the ER-negative MDA-MB-435s human breast cancer xenografts in SCID mice [10]. Moreover, 2-MeO-E_2 has strong antiangiogenic effects in vitro and in vivo at pharmacological concentrations [9,10]. Notably, the antiangiogenic effect of 2-MeO-E_2 in cultured

Abbreviations: E_2, 17β-estradiol; 2-MeO-E_2, 2-methoxyestradiol; ER, estrogen receptor; COMT, catechol-*O*-methyltransferase; EMEM, Eagle's modified minimum essential medium; IMEM, Iscove's modified minimum essential medium; HPLC, high-pressure liquid chromatography

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vascular endothelial cells was not shared by several of its close structural analogs (such as 2-MeO-E_1 [9]), suggesting a high degree of specificity for this action.

Because of the intriguing antiproliferative, apoptotic, and antiangiogenic actions of 2-MeO-E_2 and also because of its presumed low systematic toxicity, considerable research efforts have been initiated lately to explore the usefulness of 2-MeO-E_2 as a low-toxicity chemotherapeutic agent for human breast cancer as well as for other cancers (discussed in [6]). In the present study, we compared the effects of 2-MeO-E_2 on the growth of two representative ER-negative human breast cancer cell lines (MDA-MB-231 and MDA-MB-435s) and two representative ER-positive human breast cancer cell lines (MCF-7 and T-47D). We report here our findings that 2-MeO-E_2, in a concentration-dependent manner, exerted both mitogenic and antiproliferative actions in the ER-positive human breast cancer cells, but its mitogenic action was not observed in the ER-negative human breast cancer cells. We believe that this finding is of timely importance to the on-going clinical trials designed to evaluate the effectiveness of 2-MeO-E_2 for the treatment of different types of human breast cancer.

2. Materials and methods

2.1. Chemicals

2-MeO-E_2 and 2-hydroxy-E_2 were purchased from Steraloids (Newport, RI). Our HPLC analysis showed that 2-MeO-E_2 from Steraloids only had ∼94% purity, but no E_2 or estrone was detected. We re-purified the 2-MeO-E_2 with HPLC when it was used in all the cell culture experiments described in this paper. ICI-182,780 was obtained from AstraZeneca Co. (Wilmington, DE). Insulin, E_2, crystal violet, 50% glutaradehyde, dithiothreitol, Triton X–100, dextran-coated charcoal, and fetal bovine serum (FBS) were obtained from the Sigma Chemical Co. (St. Louis, MO). The antibiotics solution (containing 10,000 U/ml penicillin and 10 mg/ml streptomycin), trypsin–versene mixture (containing 0.25% trypsin and 0.02% EDTA), RPMI-1640 medium (phenol red-free), Eagle's modified minimum essential medium (EMEM; phenol red-free), Iscove's modified minimum essential medium (IMEM), and calf bovine serum were purchased from Life Technology (Rockville, MD). The recombinant human ERα and ERβ proteins were obtained from PanVera Corporation (Madison, WI). Hydroxylapatite (HAP) was obtained from Calbiochem (San Diego, CA). [2,4,6,7,16,17-^{3}H]E_2 was obtained from NEN Life Sciences (Boston, MA).

2.2. Culture of human breast cancer cell lines

Two ER-positive human breast cancer cell lines (MCF-7 and T-47D) and two ER-negative human breast cancer cell lines (MDA-MB-435s and MDA-MB-231) were obtained from the American Type Culture Collection (ATCC) (Manassas, VA). The MCF-7 cells were propagated in the phenol red-free EMEM supplemented with 10% fetal bovine serum, 1 mM pyruvate, 0.1 mM nonessential amino acids, 1.5 g/l sodium bicarbonate, and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). The T-47D cells were grown in RPMI-1640 medium (phenol red-free) supplemented with 10% fetal bovine serum and 1% antibiotics (containing 100 U/ml penicillin and 100 μg/ml streptomycin). IMEM, supplemented with 10% fetal bovine serum and the same amount of antibiotics, was used for culturing the ER-negative MDA-MB-231 and MDA-MB-435s cells. Unless otherwise indicated, these cell culture media were used for the experiments described in this paper.

The human breast cancer cells were first propagated in the 75 cm^2 flasks under 37 °C air with 5% CO_2 and 95% humidity. After reaching subconfluence, they were detached from the flask by treatment with 3 ml of the trypsin–EDTA solution for ∼5 min. During this brief digestive enzyme treatment, the cells were frequently monitored under a microscope to avoid over digestion of the cells. Immediately following this step, 5 ml of the whole medium (containing 10% fetal bovine serum) was added to terminate the digestion, and the detached cells were thoroughly dispersed with gentle agitation. Cell suspensions were centrifuged and cell sediments were re-suspended in the culture medium at the desired 10^5 cells/ml density. A 0.1 ml aliquot of the cell suspension was then added to each well of the 96-well microplate at a final density of 5×10^3 or 10^4 cells per well. After the cells were allowed to attach and grow for 48 h, the cell culture medium was changed and different treatments were also given at that time. Unless otherwise indicated, the whole medium (as described above) was used in most experiments. The drug treatment lasted for 6 days with one medium change on the fourth day following the initial drug treatment.

It should be noted that in some of the experiments that were designed to study the mitogenic effect of 2-MeO-E_2 or E_2 in the ER-positive MCF-7 cells, we used the EMEM supplemented with 5% of charcoal-stripped calf bovine serum. The charcoal-stripping procedure was employed to remove the endogenous estrogens present in the serum. Preparation of charcoal-stripped serum was done by following a method described earlier [20] with minor modifications. Briefly, the serum was mixed with 1% (w/v) dextran-coated charcoal and incubated at 56 °C for 1 h with constant mild stirring, and then the charcoal was removed by centrifugation at $27{,}500 \times g$ for 30 min. The same charcoal-stripping procedure was repeated a second time, and then the supernatant was filtered through a 0.22 μm filter (Millipore Corporation, Bedford, MA).

2.3. Preparation of 2-MeO-E_2 solutions

Due to the high lipophilicity of 2-MeO-E_2, its stock solution (at a 20 mM concentration) was prepared in pure ethanol

(200 proof). The stock solution was then filtered with a 0.22 μm pore size Millex syringe filter, and the filtrates were stored at −20 °C in a tightly-sealed sterile tube. Shortly before introducing 2-MeO-E_2 to the cultured cells, it was freshly diluted with sterilized phosphate buffered saline to desired concentrations (usually at 20 μM), and the mixture was thoroughly vortexed and also sonicated for ∼1 min. The 2-MeO-E_2 solution was then mixed with the culture medium and added to each well, giving the final concentration of ethanol in the cell culture medium of <0.01%. Similarly, the stock solutions of E_2 and ICI-182,780 (at concentrations of 20 mM each) were also prepared in pure ethanol. They were further diluted with phosphate buffered saline to the desired concentrations (usually <5 μM) immediately before use. Because very low final concentrations of E_2 and the ICI compound were present in the culture medium, the amount of ethanol introduced is practically negligible.

2.4. Measurement of cell growth

The cell density in the 96-well microplates was determined by using the crystal violet staining method [21]. This reliable, convenient quantification method was also used in several similar earlier studies [7,21,22]. Briefly, the culture medium in the microplates was first removed by aspiration, and then the cells in each well were fixed with 1% glutaradehyde for 15 min. After the fixation solution was removed, each well was rinsed gently with tap water and allowed to dry at room temperature. The cells in each well were then stained with 50 μl of 0.5% crystal violet (w/v, dissolved in 20% methanol and 80% deionized water) for 15 min at room temperature, and the plates were rinsed carefully with tap water to remove residual crystal violet dye. The stained dye was then dissolved in 100 μl of 0.5% Triton X–100 for overnight. After addition of 50 μl of 200-proof ethanol, the optical density values of each well were measured at 560 and 405 nm with a UVmax microplate reader (Molecular Device, Palo Alto, CA), and the difference in the optical density values at 560 and 405 nm was used to reflect the relative cell density in each well.

It is of note that in several of our initial trial experiments, we also compared the MTT cell proliferation assay with the crystal violet staining method. The MTT assay has been commonly used to quantify the in vitro cell proliferation based on measurement of the mitochondrial succinate dehydrogenase activity [23]. Through replicate determinations of the untreated control cells with both methods, we noted that the crystal violet staining method was not only rather convenient and cost far less, but it also yielded highly reproducible results. In comparison, we noted that the incubation time after addition of the MTT reagents needed to be frequently and meticulously re-adjusted for different cell lines and at different cell density in order to yield good results. Besides, the MTT assay often had larger interassay variations than the crystal violet method and failed to produce reliable results when the cell density was too high. Lastly, it is of note that the MTT assay, as was suggested earlier by other investigators [23], would be more prone to variations when the cellular mitochondrial functions were impaired by the anticancer agents like 2-MeO-E_2. For these reasons, we had chosen to use the crystal violet staining method in the present study.

2.5. Measurement of the relative binding affinity (RBA) for human ERα and ERβ

The following buffer solutions were used in the ER binding assays, and they were prepared beforehand and stored at 4 °C. The binding buffer consisted of 10% glycerol, 2 mM dithiothreitol, 1 mg/ml BSA and 10 mM Tris–HCl, pH 7.5. The ERα washing buffer contained 40 mM Tris–HCl and 100 mM KCl (pH 7.4), but the ERβ washing buffer contained only 40 mM Tris–HCl (pH 7.5). The 50% hydroxylapatite (HAP) slurry was prepared first by vigorously mixing 10 g HAP with 60 ml of the Tris–HCl solution (50 mM, pH 7.4). HAP was then allowed to settle for 20 min, and the supernatant was decanted. The above procedures were repeated 12 times, and afterwards the HAP was kept in the 50 mM Tris–HCl solution overnight at 4 °C. The HAP slurry was then adjusted to a final concentration of 50% (v/v) by using the same Tris–HCl solution and stored at 4 °C. The HAP slurry was stable for several months.

On the day of conducting the ER binding assay, [^{3}H]E_2 solution (at 22.22 nM) was freshly diluted in the binding buffer, and aliquots (45 μl) of the [^{3}H]E_2 solution were added to 1.5 ml microcentrifuge tubes. Then 50 μl of varying concentrations (0.06, 0.24, 0.98, 3.9, 15.6, 62.5, 250 and 1000 nM) of the competing ligand was added. The ERα or ERβ protein was freshly diluted in the binding buffer and mixed gently with repeated pipettings, and an aliquot (5 μl) was then added and mixed gently. Nonspecific binding by the [^{3}H]E_2 was determined by addition of a 400-fold concentration of the nonradioactive E_2. The binding mixture was incubated at room temperatures for 2 h. At the end of the incubation, 100 μl of the HAP slurry was added and the tubes were incubated on ice for 15 min with three times of brief vortexing. Aliquot (1 ml) of the appropriate wash buffer was then added, mixed, and centrifuged at 10,000 × g for 1 min, and the supernatants were discarded. This wash step is repeated twice. The HAP pellets were then resuspended in 200 μl ethanol (followed by another rinse with 200 μl ethanol), and then the content was transferred to scintillation vials (containing 4 ml of the scintillation fluid) for measurement of ^{3}H-radioactivity with a liquid scintillation counter (Packard Tri-CARB 2900 TR; Downers Grove, IL).

2.6. Statistical analysis

The rate of cell growth in the control or drug-treated groups was expressed as mean ± S.E. of the values obtained usually from five to seven replicate wells. The point estimate and 95% confidence interval (CI) of the IC_{50} values

were calculated according to the equation for sigmoidal dose–response curves (with variable slopes) by using the non-linear regression curve-fitting model of the Prism software. Unless otherwise indicated, one-way ANOVA was used for multi-variables comparisons. A P value <0.05 was considered to be statistically significant, and a P value <0.01 was considered statistically very significant.

3. Results

3.1. Concentration-dependent dual actions of 2-MeO-E_2 on the growth of ER-positive human breast cancer cells

To evaluate the effects of 2-MeO-E_2 on the growth of two representative ER-positive human breast cancer cell lines (MCF-7 and T-47D), we used a cell culture medium supplemented with 10% of fetal bovine serum (not charcoal-stripped) and 10 nM of exogenous E_2. Under this estrogen-rich culture condition, we found that the ER-positive MCF-7 and T-47D cells had a similar sensitivity to 2-MeO-E_2's antiproliferative action. 2-MeO-E_2 inhibited the growth of MCF-7 and T-47D cells in a concentration-dependent manner (Fig. 1), with IC_{50} values of 0.81 μM (95% CI = 0.72–0.90 μM, $R^2 = 0.9959$) and 1.29 μM (95% CI = 1.25–1.34 μM, $R^2 = 0.9961$), respectively. A near complete growth inhibition of these two cell lines was obtained when 2 μM of 2-MeO-E_2 was present.

Interestingly, when the exogenous E_2 (10 nM) was removed from the same cell culture medium, we observed that 2-MeO-E_2 at relatively low concentrations (from 10 to 750 nM) exerted a weak mitogenic effect (Fig. 1). A comparison of the cell growth rates in the presence versus absence of 10 nM of exogenous E_2 indicated that the apparent efficacy of 2-MeO-E_2's mitogenic action in MCF-7 and T-47D cells was 75–80% of that exerted by 10 nM of exogenous E_2. However, further increasing the concentrations of 2-MeO-E_2 from 750 nM to 2 μM produced a concentration-dependent antiproliferative effect in these two ER-positive cell lines. It should be noted that the overall curve patterns for the concentration-dependent growth inhibition by 2-MeO-E_2 in the presence or absence of exogenous E_2 were quite similar (Fig. 1).

To further characterize the efficacy and potency of 2-MeO-E_2's mitogenic action in the ER-positive human breast cancer cells, we used MCF-7 cells as a representative cell line and cultured these cells in the phenol red-free EMEM supplemented with charcoal-stripped calf bovine serum without the exogenous E_2. The MCF-7 cells were first cultured under this estrogen-deficient condition for 48 h and then they were treated with different concentrations of 2-MeO-E_2 and/or E_2. Fresh cell culture medium containing 2-MeO-E_2 and/or E_2 was refurbished every other day. The cell density after different lengths of treatment with 2-MeO-E_2 and/or E_2 was determined.

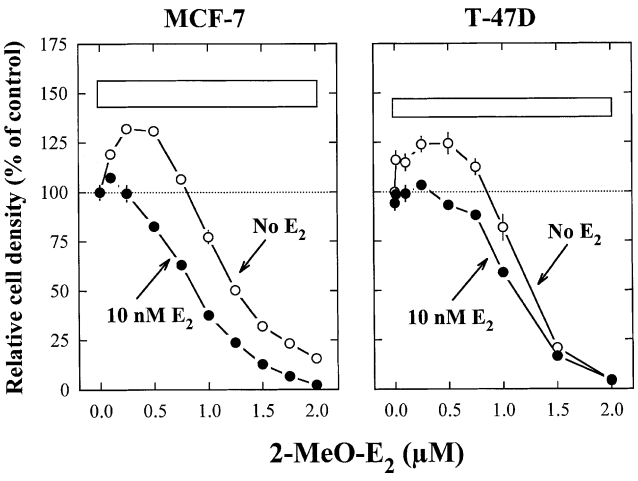


Fig. 1. Effects of 2-MeO-E_2 on the growth of ER-positive MCF-7 and T-47D cells in the presence or absence of 10 nM E_2. The methods for culturing these ER-positive cancer cells were described in Section 2. Forty-eight hours after an aliquot of the cell suspension (containing 10^4 cells) was placed into the 96-well microplate, the culture medium was changed and different concentrations of 2-MeO-E_2 or E_2 (dissolved in phosphate buffer) were introduced at that time. Note that the following concentrations of 2-MeO-E_2 were used for MCF-7 cells: 0, 0.1, 0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75 and 2 μM, and concentrations used for T-47D cells were: 0, 0.01, 0.1, 0.25, 0.5, 0.75, 1, 1.25, 1.5, and 2 μM. Each of the drug treatments lasted for 6 days with one medium change on the fourth day following the initial drug treatment. Cell density in each well was determined by using the crystal violet staining method followed by spectrometric measurement with a UVmax microplate reader, and the starting cell density immediately prior to estrogen treatment was subtracted. For comparison, the rate of cell growth in the absence of 2-MeO-E_2 was arbitrarily assigned to be 100%, and the rate of cell growth in the presence of 2-MeO-E_2 was expressed as "% of control." Note that in the presence of 10 nM of E_2, the basal growth rates (without 2-MeO-E_2) of MCF-7 and T-47D cells was 47 ± 5 and 42 ± 4%, respectively, faster than their growth rates in the absence of E_2 (represented by the empty boxes). Each point is the mean ± S.E. of five to seven replicate measurements.

Our data showed that treatment of the estrogen-starved MCF-7 cells with 0.1, 0.5, or 2 nM of E_2 for 8 days very strongly increased the rate of cell growth by ~200% over the control (Fig. 2A). However, further increasing the concentrations of E_2 to 10 nM produced a slightly reduced mitogenic effect ($P < 0.01$) in these cells compared with the effect exerted by 0.1 or 0.5 nM of exogenous E_2 (Fig. 2A). This experiment was repeated multiple times and similar results were observed. Similarly, when the estrogen-starved MCF-7 cells were treated with 0.01 or 0.1 μM of 2-MeO-E_2 alone for 8 days, the rate of cell growth was markedly increased over the control, and the maximal mitogenic effect was ~80% of that exerted by 0.5 nM of E_2 (Fig. 2B). However, when the concentration of 2-MeO-E_2 was increased to 1 μM, only a very weak mitogenic effect was detected; when the concentration of 2-MeO-E_2 was further increased to 10 μM, strong antiproliferative and cytotoxic effects were observed (Fig. 2B). Our additional data showed that the apparent strong mitogenic effect of 2-MeO-E_2 observed at 0.01 and 0.1 nM concentrations were almost completely masked

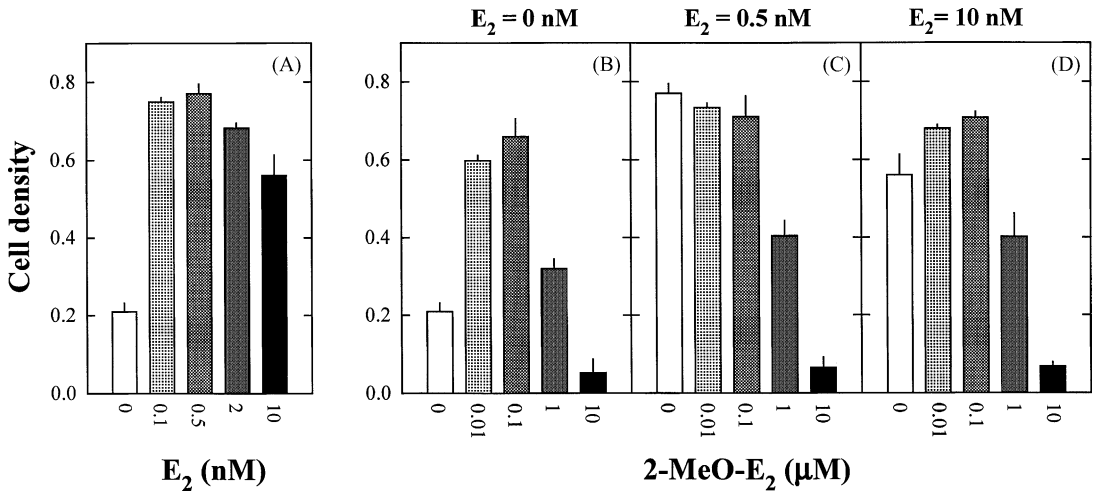


Fig. 2. Effects of 2-MeO-E_2 on the growth of estrogen-starved MCF-7 cells in the presence or absence of E_2. The MCF-7 cells were first cultured in the phenol red-free EMEM supplemented with a low concentration (5%) of the charcoal-stripped calf bovine serum and in the absence of exogenous E_2 for 48 h and then they were treated with different concentrations of 2-MeO-E_2 and/or E_2. Fresh cell culture medium containing 2-MeO-E_2 and/or E_2 was refurbished every other day. The cell density after 8 days of treatment with 2-MeO-E_2 and/or E_2 was determined by using the crystal violet staining followed by spectrometric measurement with a UVmax microplate reader. Each point is the mean ± S.E. of five to seven replicate measurements.

when 0.5 or 10 nM of exogenous E_2 was concomitantly present in the culture medium with 2-MeO-E_2 (Fig. 2C and D). This observation is consistent with the suggestion that 2-MeO-E_2's mitogenic effect is mediated by the ER.

Some additional data on the effect of E_2 (at 0.1 or 1 nM) or 2-MeO-E_2 (at 0.01, 0.1, 1, or 10 μM) or their combinations on the growth of estrogen-starved MCF-7 cells following varying lengths of treatment were shown in Fig. 3. We found that the rate of cell growth in the charcoal-stripped calf bovine serum in the absence of exogenous E_2 or 2-MeO-E_2 was rather slow. However, when the MCF-7 cells were cultured in the presence of E_2 alone (at 0.1 or 1 nM; Fig. 3B and C, open circles) or 2-MeO-E_2 alone (at 0.01 or 0.1 μM, Fig. 3A), the rate of their growth was markedly higher than that of the controls, and the mitogenic effect of E_2 or 2-MeO-E_2 was most pronounced after 8 days of treatment (Fig. 3). The antiproliferative and cytotoxic actions exerted by a very high concentration (10 μM) of 2-MeO-E_2 was not affected by the presence or absence of exogenous E_2 (Fig. 3).

To provide further support for the our suggestion that the mitogenic effect of 2-MeO-E_2 is ER-mediated, we compared the effect of ICI-182,780 (a pure antiestrogen [24,25]) in MCF-7 cells treated with E_2 or 2-MeO-E_2. In this experiment, 0.5 nM of E_2 or 100 nM of 2-MeO-E_2 was used to

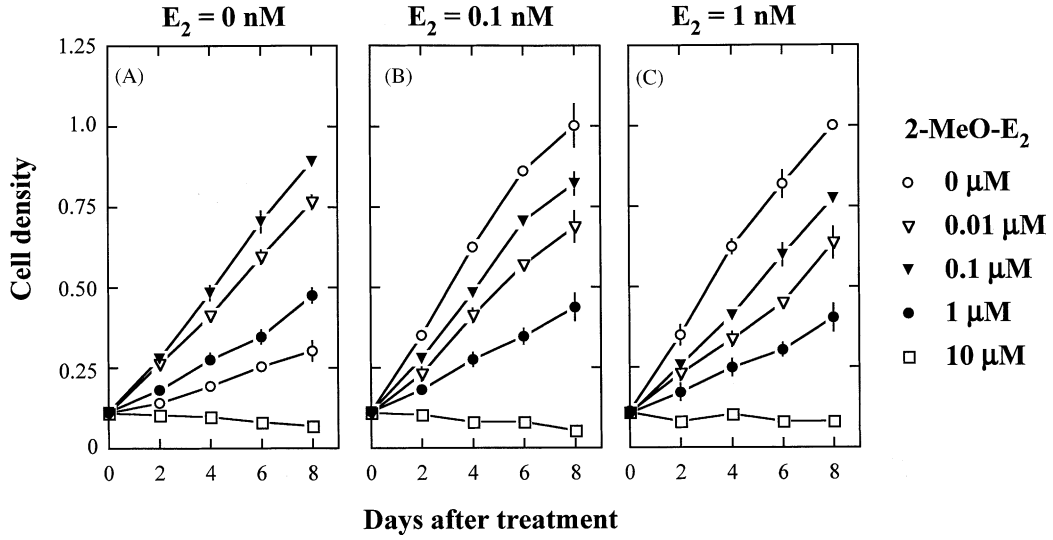


Fig. 3. Comparison of the mitogenic effect of 2-MeO-E_2 with E_2 in estrogen-starved MCF-7 cells at different time points. The experimental method was the same as described in Fig. 2. Each point is the mean ± S.E. of five to seven replicate measurements.

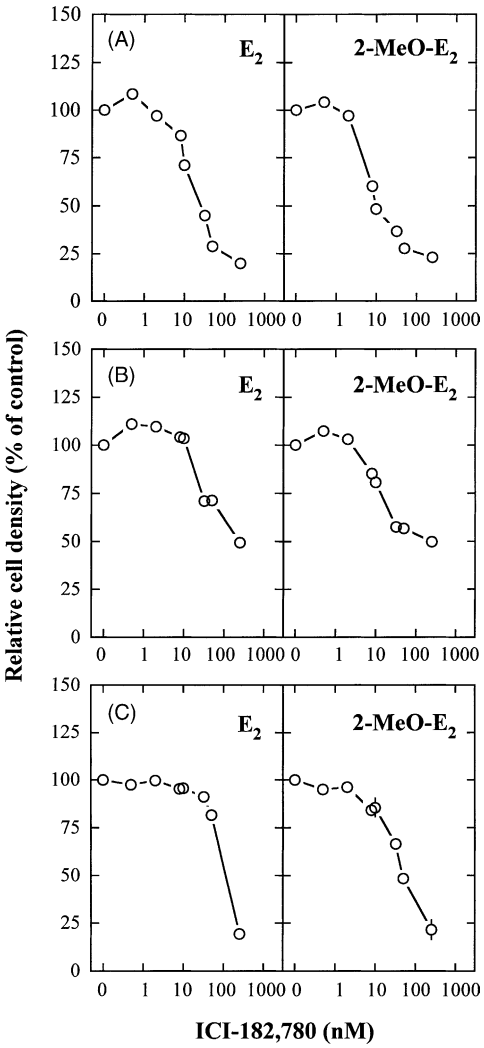


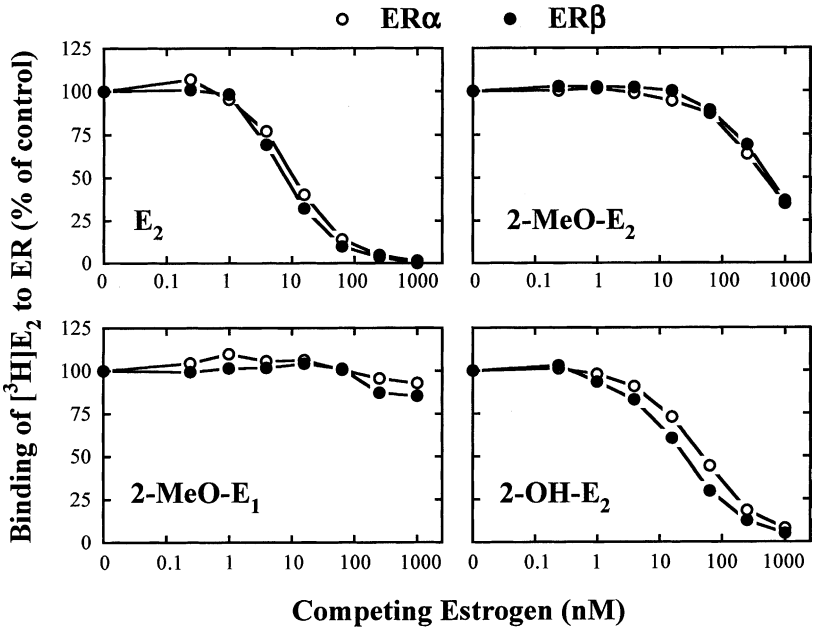
Fig. 4. Antagonistic effects of ICI-182,780 on the mitogenic effects of 2-MeO-E_2 or E_2 in the ER-positive MCF-7 cells. Cells were first cultured in the EMEM supplemented with 5% charcoal-stripped calf bovine serum (an estrogen-deficient condition), and then the cells were treated with 2-MeO-E_2 or E_2 in the absence or presence of ICI-182,780. In order to alter the sensitivity of MCF-7 cells to estrogen's growth stimulation, three slightly different culture conditions were used (data shown in panels A, B, and C). For panel A, the MCF-7 cells were initially seeded at a density of 10,000 cells/well in the 96-well plate (in the absence of exogenous insulin), and treatment of cells with an estrogen and/or an antiestrogen lasted for 6 days. For panel B, the cells were initially seeded at 5000 cells/well (in the presence of 10 μg/ml of exogenous insulin), and treatment of cells with an estrogen and/or an antiestrogen lasted only for 3 days. For panel C, the cells were initially seeded at 5000 cells per well (in the presence of 1 μg/ml of exogenous insulin), and treatment of cells with an estrogen and/or an antiestrogen lasted for 8 days. Each point is the mean ± S.E. of four replicate measurements.

produce a near maximal growth stimulation of the MCF-7 cells. Moreover, different concentrations of insulin (a known growth stimulator in MCF-7 cells [25–28]) were used to produce different basal rates of growth in these cells. A representative data set is shown in Fig. 4. We found that ICI-182,780 exerted a similar concentration-dependent inhibition of the growth of MCF-7 cells treated with 2-MeO-E_2 (100 nM) or E_2 (0.5 nM) under different growth conditions. The curve patterns for the concentration-dependent growth inhibition by ICI-182,780 as well as the degree of the maximum inhibition were almost identical either with 2-MeO-E_2 or E_2 (Fig. 4). These data further add to the evidence that the mitogenic effect of 2-MeO-E_2 in the ER-positive MCF-7 cells was mediated by the ER.

To determine whether the estrogenic activity of 2-MeO-E_2 is partially attributable to the metabolic demethylation of 2-MeO-E_2 to 2-hydroxyestradiol (a weak but more estrogenic metabolite than 2-MeO-E_2), we used HPLC to measure the amount of 2-hydroxy-E_2 released into the culture medium after the cells were cultured in the presence of 1 μM of 2-MeO-E_2 (containing ~0.4 μCi [4-^{3}H]2-MeO-E_2) for 8 days. The whole medium from each well was collected in a microcentrifuge tube and dried under a stream of nitrogen. The resulting residues were resuspended in 70 μl of pure methanol, and an aliquot (50 μl) was injected into the HPLC for analysis of 2-MeO-E_2 and its metabolite compositions. Our results showed that the major metabolite detected with MCF-7 cells was the sulfated 2-MeO-E_2, and no detectable 2-hydroxy-E_2 (conjugated or unconjugated) was present in the culture medium during the 8 days of culture. Also, little or no 2-methoxyestrone was detected with this cell line. All the other more polar metabolites combined only accounted for <3% of the total radioactivity detected. This observation clearly suggested that the estrogenic activity seen in 2-MeO-E_2-treated ER-positive cells was not attributable to its metabolic conversion to 2-hydoxy-E_2.

To provide further evidence that the mitogenic activity of 2-MeO-E_2 was largely due to its own residual estrogenic activity at the human ERs, we also determined in the present study the binding affinities of 2-MeO-E_2, 2-methoxyestrone, and 2-hydroxy-E_2 for human ERα and ERβ. Our results confirmed that 2-MeO-E_2 retained weak ER binding affinity for both human ERα and ERβ, approximately 1–2% of the binding affinity of E_2 (Fig. 5). Moreover, the binding affinity of 2-MeO-E_2 for human ERα and ERβ was significantly weaker than 2-hydroxy-E_2. In comparison, 2-methoxyestrone had little binding activity for the human ERα and ERβ (Fig. 5).

In summary, in the absence of exogenous E_2, 2-MeO-E_2 at relatively low concentrations (10–750 nM) showed a moderate mitogenic effect in two ER-positive human breast cancer cell lines. The mitogenic effect of 2-MeO-E_2, like that of E_2, was magnified when the ER-positive cancer cells were pre-cultured under estrogen-deficient conditions for 48 h. However, the mitogenic effect of 2-MeO-E_2 in the ER-positive MCF-7 cells was masked when exogenous E_2 was concomitantly present in the culture medium. Moreover, 2-MeO-E_2 retained weak binding affinity for the human ERα and ERβ, and the mitogenic effect of 2-MeO-E_2 or E_2 in these cells was inhibited in a similar concentration-dependent manner by ICI-182,780, a pure ER antagonist. Taken together, these data clearly indicated



	ERα		ERβ	
	IC50 (nM)	*RBA*	*IC50* (nM)	*RBA*
E_2	11.22	100%	8.91	100%
2-MeO-E_2	501.19	2%	630.96	1%
2-MeO-E_1	--	--	--	--
2-OH-E_2	50.12	22%	25.12	36%

Fig. 5. Inhibition of the binding of 10 nM [^{3}H]E_2 to human ERα and ERβ by 2-MeO-E_2, 2-methoxyestrone (2-MeO-E_1), 2-hydroxy-E_2 (2-OH-E_2) and 17β-estradiol (E_2). Eight concentrations (0.06, 0.24, 0.98, 3.9, 15.6, 62.5, 250, and 1000 nM) of each competing estrogen were tested. The IC_{50} values for each competing estrogen was calculated according to the sigmoid inhibition curves, and the relative binding affinity (RBA) for each test compound was calculated against E_2 by using the following equation: RBA = IC_{50} for E_2/IC_{50} for the test compound. Each point was the mean of duplicate measurements, with average variations <5%.

that 2-MeO-E_2's mitogenic effect in the ER-positive cancer cells was attributable to its residual estrogenic activity. However, at relatively higher concentrations, 2-MeO-E_2 exerted a predominant, concentration-dependent antiproliferative effect in the ER-positive MCF-7 and T-47D cells, regardless of whether exogenous E_2 was present or not.

3.2. Antiproliferative actions of 2-MeO-E_2 in ER-negative human breast cancer cells

To compare the effects of 2-MeO-E_2 in the ER-negative MDA-MB-231 and MDA-MB-435s human breast cancer cells, these cells were also cultured in a medium supplemented with fetal bovine serum with or without 10 nM of exogenous E_2. The effect of 2-MeO-E_2 at different concentrations (from 10 nM to 2 μM) on the growth of these two cell lines were shown in Fig. 6. Regardless of whether 10 nM of exogenous E_2 was present or not, 2-MeO-E_2 showed almost identical antiproliferative effect in each of the two ER-negative cell lines. The IC_{50} value of 2-MeO-E_2 in MDA-MB-435s cells in the absence of 10 nM of exogenous E_2 was 0.87 μM (95% CI = 0.82–0.91 μM, $R^2 = 0.9973$), and the IC_{50} value in the presence of 10 nM E_2 was 0.81 μM (95% CI = 0.78–0.84, $R^2 = 0.9986$ μM). A near complete growth inhibition of MDA-MB-435s cells was obtained when 2 μM of 2-MeO-E_2 was present. In comparison, the MDA-MB-231 cells were relatively less sensitive to the antiproliferative actions of 2-MeO-E_2. The IC_{50} values of 2-MeO-E_2 in MDA-MB-231 cells in the absence of 10 nM of exogenous E_2 were 1.38 μM (95% CI = 1.29–1.46 μM, $R^2 = 0.9915$), and the IC_{50} value in the presence of 10 nM E_2 were 1.34 μM (95% CI = 1.29–1.46 μM, $R^2 = 0.9974$). Notably, only ~75% of growth inhibition of MDA-MB-23 cells was obtained when 2 μM of 2-MeO-E_2 was present.

In summary, 2-MeO-E_2 had a concentration-dependent antiproliferative effect in two representative ER-negative human breast cancer cell lines tested. As expected, the presence or absence of exogenous E_2 (up to 10 nM concentrations) in the cell culture medium did not affect the potency and efficacy of 2-MeO-E_2's antiproliferative action in these two cell lines because these cells are ER-negative.

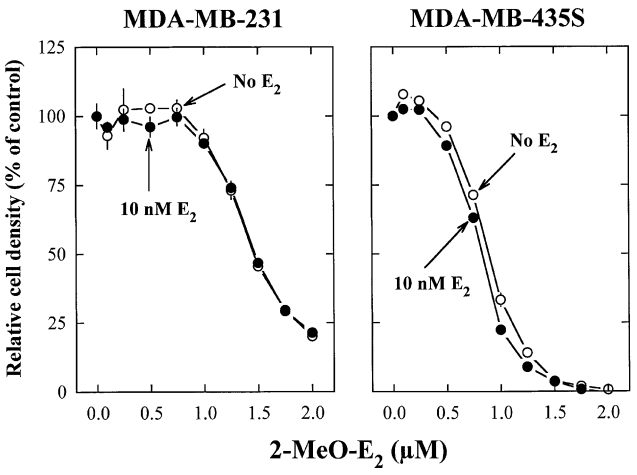


Fig. 6. Effects of 2-MeO-E_2 on the growth of ER-negative MDA-MB-231 and MDA-MB-435s cells in the presence or absence of 10 nM E_2. Forty-eight hours after an aliquot of the cell suspension (containing 10^4 cells) was placed into the 96-well microplate, the culture medium was changed and different concentrations of 2-MeO-E_2 or E_2 (dissolved in the medium) were introduced at that time. Each of the drug treatments lasted for 6 days with one medium change on the fourth day following the initial drug treatment. Cell density in each well was determined by using the crystal violet staining method followed by spectrometric measurement with a UVmax microplate reader, and the starting cell density immediately prior to estrogen treatment was subtracted. For comparison, the rate of cell growth in the absence of 2-MeO-E_2 was arbitrarily assigned to be 100%, and the rate of cell growth in the presence of 2-MeO-E_2 was expressed as "% of control." Note that in the presence of 10 nM of E_2, the basal growth rates (without 2-MeO-E_2) of these two cell lines was almost the same as their growth rates in the absence of E_2. Each point is the mean ± S.E. of five to seven replicate measurements.

4. Discussion

In the present study, we compared the effects of 2-MeO-E_2 on the growth of two ER-negative and two ER-positive human breast cancer cell lines. Our results showed that 2-MeO-E_2 had a concentration-dependent antiproliferative effect in the ER-negative MDA-MB-231 and MDA-MB-435s human breast cancer cells, and the average IC_{50} values of 2-MeO-E_2 were 0.84 and 1.36 μM, respectively. The presence or absence of exogenous E_2 in the cell culture medium did not affect 2-MeO-E_2's antiproliferative action in these two ER-negative cell lines. When the ER-positive MCF-7 and T-47D cells were cultured in a medium supplemented with 10 nM of exogenous E_2, 2-MeO-E_2 at 750 nM to 2 μM concentrations exerted a similar concentration-dependent antiproliferative effect. The IC_{50} values and the overall curve patterns for the growth inhibition of these two ER-positive cell lines by 2-MeO-E_2 were similar to those observed with two ER-negative cell lines, and are also in close agreement with the results reported by others [11,12,19].

However, when the ER-positive human breast cancer cell lines were cultured in the absence of exogenous E_2, we observed that 2-MeO-E_2 at relatively low concentrations (10–750 nM) exerted a mitogenic effect in these cells. The maximal mitogenic effect was ~80% of that of E_2. We believe that this mitogenic effect of 2-MeO-E_2 is attributable to its residual estrogenic activity on the basis of the following experimental observations made in our present study: (i) The mitogenic effect of 2-MeO-E_2 was only manifested in the two ER-positive human breast cancer cell lines but not in the two ER-negative cancer cell lines. (ii) After the ER-positive MCF-7 cells were pre-cultured in estrogen-deficient conditions for 48 h, the sensitivity of these cells to the mitogenic actions of 2-MeO-E_2 and E_2 was increased in a parallel manner. (iii) The apparent mitogenic effect of 2-MeO-E_2 in the ER-positive cells was partially or fully masked when exogenous E_2 was concomitantly present in the culture medium. (iv) The mitogenic effect of 2-MeO-E_2 or E_2 was inhibited in a similar concentration-dependent manner by ICI-182,780, a pure ER antagonist. (v) We confirmed that 2-MeO-E_2 retained weak and readily detectable binding affinity for both human ERα and ERβ, although its binding affinity was weaker than 2-OH-E_2. (vi) We detected little 2-hydroxy–E_2 or its sulfate/glucuronide conjugates formed in the culture medium when the MCF-7 cells were incubated with [4-^{3}H]2-MeO-E_2 (1 μM) for up to 8 days. Although Zhu et al. [29] earlier showed that 2- or 4–MeO–E_2 could be readily converted to catechol estrogens by the NADPH-dependent cytochrome P450 enzymes, this observation suggests that metabolic formation of 2-hydroxy–E_2 (a mildly estrogenic metabolite) from 2-MeO-E_2 did not contribute appreciably to the estrogenic activity of 2-MeO-E_2 in the cultured MCF-7 cells. Notably, it was recently suggested [19] that 2-MeO-E_2 per se was not estrogenic and the estrogenic activity observed with 2-MeO-E_2 administration likely was due to its oxidative metabolism (mainly by cytochrome P450 enzymes) to other unknown estrogenic metabolite(s). We think that it is very unlikely that any other hydroxylated or keto metabolites of 2-MeO-E_2 would be more estrogenic than 2-MeO-E_2 in light of the fact that none of the known oxidative metabolites of E_2 is more estrogenic than E_2. On the basis of all the evidence obtained from this study, it is quite clear to us that the mitogenic effect of 2-MeO-E_2 in the ER-positive breast cancer cells is mediated by the ERs and is primarily attributable to 2-MeO-E_2's residual estrogenic activity.

Our results also showed that while 0.5 nM E_2 produced a near maximal mitogenic effect in the estrogen-starved MCF-7 cells (Fig. 2), 2-MeO-E_2 at ~100 nM concentrations exerted a maximal mitogenic action in these cells. This data would suggest that the estrogenic *potency* of 2-MeO-E_2 is roughly 0.5% of that of E_2 at the human ER. This estimate of 2-MeO-E_2's relative estrogenic potency in the ER-positive human breast cancer cells is roughly in agreement with the biochemical estimates of 2-MeO-E_2's relative binding affinity for the rat uterine ER or human ERα. An earlier studies indicated that the binding affinity of 2-MeO-E_2 for the rat uterine ER is roughly 0.1–0.5% of that of E_2 [3]. Data from our present study showed that the relative binding affinity

(*RBAs*) of 2-MeO-E_2 for ERα and ERβ is 1–2% of that of E_2, which is slightly higher than the values reported in the recent study which showed the *RBA* of 2-MeO-E_2 for human ERα and ERβ was 0.2 and 0.03% of E_2, respectively [19]

Notably, our results also showed that the apparent *efficacy* of the ER-mediated mitogenic action of 2-MeO-E_2 was 75–80% of that E_2. Mechanistically, it is possible that the apparent inability of 2-MeO-E_2 to elicit the same maximal mitogenic effect as that of E_2 might have been partially due to the intrinsic antirpoliferative action of 2-MeO-E_2, which could *functionally* antagonize its mitogenic effect. This possibility, however, was somewhat weakened by our observation that 2-MeO-E_2 at 10–750 nM concentrations (which produced a strong growth stimulation) did not have substantial antiproliferative effect in the two ER-negative cancer cell lines tested. Accordingly, the possibility cannot be completely ruled out that 2-MeO-E_2 may, in fact, be a partial agonist at the classical ER.

It is well known that tamoxifen or ICI-182,780 can inhibit the estrogen-dependent growth of the ER-positive human breast cancer cells by antagonizing estrogen's actions [24]. However, it is also known that E_2 itself at high concentrations could inhibit the growth of human breast cancer cells, apparently through ER-mediated signaling pathways [30–32]. Here it needs to be pointed out that while the weak growth-stimulatory effect of 2-MeO-E_2 is mediated by the ER, its predominant growth-inhibitory effect at high concentrations is not believed to be mediated by ER-signaling pathways. This suggestion is consistent with the results from our present study showing that 2-MeO-E_2 had a highly-similar growth-inhibitory effect in both ER-positive and ER-negative cells, and this effect was not altered by the presence or absence of exogenous E_2, and it is also in line with some of the earlier mechanistic studies which suggested that 2-MeO-E_2 was an apoptotic agent likely through the disruption of microtubule functions [6,11–17].

Given the fact that larger-scale clinical trials are presently underway to evaluate the effectiveness of 2-MeO-E_2 as an anticancer agent for both ER-positive and ER-negative human breast cancers, the timely observations described in our present study may be of considerable clinical relevance. According to the ER status, a human breast cancer is usually classified either as an ER-positive cancer or as an ER-negative cancer. For the ER-negative breast cancer, our results showed that 2-MeO-E_2 only had a consistent antiproliferative effect and no mitogenic action was observed. Therefore, 2-MeO-E_2 appears to have no apparent contraindications for its clinical use in the ER-negative human breast cancers. In line with this suggestion, an earlier study has shown that 2-MeO-E_2 at pharmacological doses effectively inhibited the growth of the ER-negative MDA-MB-453s human breast cancer xenografts in immune-suppressed mice [10]. However, because of the mitogenic actions of 2-MeO-E_2 in the ER-positive human breast cancer cells as demonstrated in the present study, cautions are advised when 2-MeO-E_2 is considered for the treatment of ER-positive human breast cancer. Moreover, since the use of an ER antagonist [33–35] to block estrogen's action at the receptor or the use of an aromatase inhibitor [35–38] to inhibit the biosynthesis of endogenous estrogens is commonly prescribed for the ER-positive human breast cancer, such chronic antiestrogen therapy is expected to diminish estrogenic stimulus in the breast cancer cells, and subsequently may lead to their sensitization to estrogenic hormones. In the light of the observations made in the present study, estrogen starvation of the ER-positive human breast cancer cells would markedly sensitize their response to the mitogenic actions of 2-MeO-E_2, and thereby may produce unwanted strong growth stimulation in these cancer cells. Here it is also of note that since 2-MeO-E_2's mitogenic action is mediated by the ER, this effect may be partially blocked off when the antiestrogen is an ER antagonist. In comparison, if the antiestrogen is an aromatase inhibitor, then 2-MeO-E_2's mitogenic actions in the sensitized breast cancer cells would not be effectively protected for.

In summary, the results of our present study demonstrated that while 2-MeO-E_2 has a consistent antiproliferative effect in the ER-negative human breast cancer cells, it has both mitogenic and antiproliferative actions in the ER-positive human breast cancer cells (summarized in Fig. 7). While the antiproliferative effect of 2-MeO-E_2 is independent of the ER status, the mitogenic action of 2-MeO-E_2 in the ER-positive cancer cells is mediated by the ER and is largely attributable to 2-MeO-E_2's residual intrinsic estrogenic activity. We believe that these findings are of timely importance to the on-going clinical trials designed to evaluate 2-MeO-E_2's effectiveness in different types of human

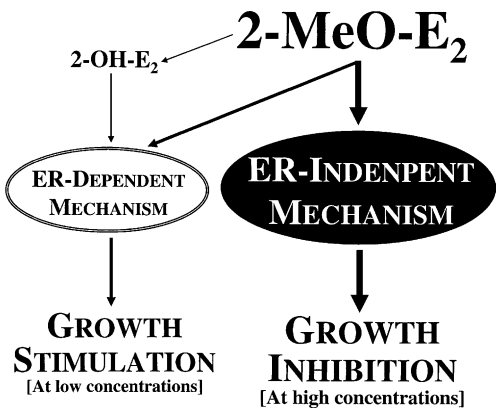


Fig. 7. Schematic illustration of the dual actions of 2-MeO-E_2 on the growth of ER-positive human breast cancer cells. At relatively low concentrations, 2-MeO-E_2 has a mitogenic effect, which is mediated by the ER and is mainly attributable to its weak intrinsic estrogenic activity. When 2-MeO-E_2 is administered in vivo, the metabolic conversion (demethylation) to 2-hydroxyestradiol (2-OH-E_2) by certain hepatic cytochrome P450 (CYP) enzymes may also add to its estrogenic activity. At high concentrations, 2-MeO-E_2 has a dominant antiproliferative effect, which is independent of the ER and is not altered by the presence or absence of an estrogen.

breast cancer. Our results may assist in the better design of clinical trials as well as the better interpretation of clinical outcomes when 2-MeO-E_2 is used either as a single agent therapy or as part of a combination therapy for different types of human breast cancer.

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