Selective Insensitivity of ZR-75-1 Human Breast Cancer Cells to 2-Methoxyestradiol: Evidence for Type II 17β-Hydroxysteroid Dehydrogenase as the Underlying Cause

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Abstract

2-Methoxyestradiol (2-MeO-E_2), a nonpolar endogenous metabolite of 17β-estradiol, has strong antiproliferative, apoptotic, and antiangiogenic actions. Among the four human breast cancer cell lines tested (MCF-7, T-47D, ZR-75-1, and MDA-MB-435s), the ZR-75-1 cells were selectively insensitive to the antiproliferative actions of 2-MeO-E_2, although these cells had a similar sensitivity as other cell lines to several other anticancer agents (5-fluorouracil, mitomycin C, doxorubicin, colchicine, vinorelbine, and paclitaxel). Mechanistically, this insensitivity is largely attributable to the presence of high levels of a steroid-selective metabolizing enzyme, the type II 17β-hydroxysteroid dehydrogenase (17β-HSD), in the ZR-75-1 cells, which rapidly converts 2-MeO-E_2 to the inactive 2-methoxyestrone, but this enzyme does not metabolically inactivate other nonsteroidal anticancer agents. The type II 17β-HSD–mediated conversion of 2-MeO-E_2 to 2-methoxyestrone in ZR-75-1 cells followed the first-order kinetics, with a very short half-life (~2 hours). In comparison, the T-47D, MCF-7, and MDA-MB-435s human breast cancer cells, which were highly sensitive to 2-MeO-E_2, had very low or undetectable catalytic activity for the conversion of 2-MeO-E_2 to 2-methoxyestrone. Reverse transcription-PCR analysis of the mRNA levels of three known oxidative 17β-HSD isozymes (types II, IV, and VIII) revealed that only the type II isozyme was selectively expressed in the ZR-75-1 cells, whereas the other two isozymes were expressed in all four cell lines. Taken together, our results showed, for the first time, that the high levels of type II 17β-HSD present in ZR-75-1 cells were largely responsible for the facile conversion of 2-MeO-E_2 to 2-methoxyestrone and also for the selective insensitivity to the antiproliferative actions of 2-MeO-E_2. (Cancer Res 2005; 65(13): 5802-11)

Introduction

Many studies have shown that 2-methoxyestradiol (2-MeO-E_2), a well-known nonpolar metabolite of the endogenous parent hormone 17β-estradiol (E_2) (reviewed in refs. 1, 2), has strong antiproliferative and apoptotic actions in a number of cultured cancer cell lines when it was present at pharmacologic concentrations (3–12). Among many of the human cancer cell lines tested thus far, several human breast cancer cell lines were found to be highly sensitive to the antiproliferative actions of 2-MeO-E_2 *in vitro* (3, 4, 12). Additional studies also showed that 2-MeO-E_2 inhibited the growth of human breast cancer cells when they were transplanted into immune-suppressed mice (10). Moreover, 2-MeO-E_2 has strong antiangiogenic effects both *in vitro* and *in vivo* at pharmacologic concentrations (9–11, 13). An earlier study revealed that some of the unique biological actions of 2-MeO-E_2 were not shared by several of its close structural analogues (such as 2-methoxy-E_1 and 4-hydroxy-E_2; ref. 10), which suggested a high degree of specificity for the actions of 2-MeO-E_2.

Because of the intriguing antiproliferative and antiangiogenic actions of 2-MeO-E_2, and also because of its presumed low toxicity, a great deal of research efforts have been initiated in the past few years to explore the usefulness of 2-MeO-E_2 as an effective, low-toxicity chemotherapeutic agent for human breast cancer as well as for other human cancers (14). In the present study, we compared the growth inhibitory effect of 2-MeO-E_2 in four human breast cancer cell lines, including three ER-positive cell lines (MCF-7, T-47D, and ZR-75-1) and one ER-negative cell line (MDA-MB-435s), to determine whether different human breast cancer cell lines have a differential sensitivity to the anticancer actions of 2-MeO-E_2. For the first time, we noted that the ZR-75-1 human breast cancer cells were highly insensitive to the antiproliferative actions of 2-MeO-E_2 (particularly when these cells were grown at a higher density), whereas the other three cell lines were all highly sensitive to 2-MeO-E_2. Additional experiments showed that the ZR-75-1 cells have a similar sensitivity to a number of commonly used anticancer agents as the other three cancer cell lines. These data suggested the existence of a selective insensitivity of the ZR-75-1 cells to 2-MeO-E_2, but not to several other commonly used anticancer agents.

During our search for the mechanism(s) underlying the selective insensitivity of the ZR-75-1 cancer cells to 2-MeO-E_2, we found that these cells contained very high activity of the type II 17β-hydroxysteroid dehydrogenase (17β-HSD), a steroid-metabolizing enzyme that catalyzes the conversion of E_2 to E_1 as well as the conversion of 2-MeO-E_2 to the biologically-inactive 2-methoxy-E_1. We believe that the high levels of the oxidative 17β-HSD present in ZR-75-1 cells are largely responsible for the selective insensitivity of these cells to 2-MeO-E_2 (the concept is depicted in Fig. 1). The present study provided detailed experimental data in support of this mechanistic explanation.

Materials and Methods

Chemicals and Reagents

2-MeO-E_2, 2-methoxy-E_1, E_2, and E_1 were purchased from Steraloids (Newport, RI). Crystal violet, 50% glutaradehyde, Triton X-100, trypsin-EDTA solution (containing 0.25% trypsin and 0.02% EDTA), bovine serum albumin (BSA), fetal bovine serum (FBS), paclitaxel, doxorubicin, mitomycin C, and 5-fluorouracil were all obtained from the Sigma

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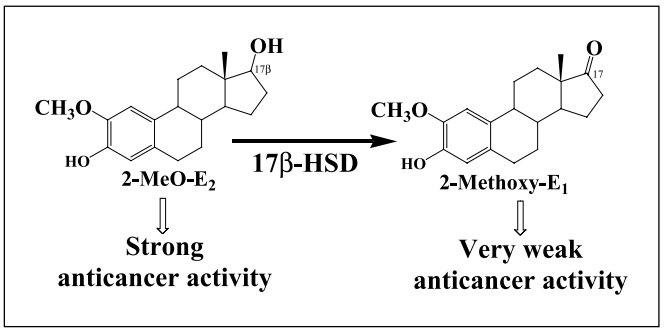

Figure 1. The proposed role of 17β-HSD in the metabolic inactivation of 2-MeO-E_2. The oxidative activity of 17β-HSD catalyzes the conversion of 17β-hydroxysteroids (such as E_2 and 2-MeO-E_2) to 17-ketosteroids (such as E_1 and 2-methoxy-E_1), whereas its reductive activity is responsible for the reverse conversion of 17-ketosteroids to 17β-hydroxysteroids. We hypothesize that the high levels of the oxidative 17β-HSD isozyme(s) present in the ZR-75-1 breast cancer cells may rapidly convert 2-MeO-E_2 to 2-methoxy-E_1 (an essentially inactive metabolite) and, thus, may contribute to the selective insensitivity to the anticancer actions of 2-MeO-E_2.

Chemical, Co. (St. Louis, MO). Vinorelbine tartrate (vinorelbine) was provided by GlaxoSmithKline (Research Triangle Park, NC). RPMI 1640 and Iscove's cell culture media and the antibiotics solution (containing 10,000 units/mL penicillin and 10 mg/mL streptomycin) were obtained from Life Technology (Rockville, MD). Spiro-δ-lactone was a generous gift from Dr. Donald Poirier at the Centre Hospitalier Universitaire de Québec (Canada).

[2,4,6,7,16,17-^{3}H]E_2 and [2,4,6,7-^{3}H]E_1 (specific activity = 118 and 100 Ci/mmol, respectively) were purchased from DuPont Life Science Products (Boston, MA). Before use in each experiment, each of the radioactive chemicals was repurified by using our high-performance liquid chromatography (HPLC) method (described later). ^{3}H-Labeled 2-MeO-E_2 was biosynthetically prepared by incubating [^{3}H]2-OH-E_2 (obtained from Moravek Biochemicals, Inc., Brea, CA) with porcine liver catechol-*O*-methyltransferase (Sigma) and *S*-adenosyl-L-methionine at 37°C for 2 hours. The incubation mixture was extracted twice with 7 mL of ethyl acetate and the extracts were dried under a stream of N_2. The radioactive 2-MeO-E_2 was isolated by using our HPLC system as described earlier (15, 16).

Culture of Human Breast Cancer Cell Lines

The three ER-positive human breast cancer cell lines (MCF-7, T-47D, and ZR-75-1) and one ER-negative human breast cancer cell line (MDA-MB-435s) were all obtained from the American Type Culture Collection (Manassas, VA). The methods for the *in vitro* culture of the ER-positive MCF-7 and T-47D cells as well as the ER-negative MDA-MB-435s cells were described in our recent study (12). For culturing the ER-positive ZR-75-1 cells, we used RPMI 1640 medium supplemented with 10% FBS and the same amount of antibiotics.

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 to ~80% confluence. They were then detached from the flask by treatment with 3 mL of the trypsin-EDTA solution for ~5 minutes. Cell suspensions were centrifuged and the cell sediments were resuspended 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 usually at a final density of 2×10^4 cells per well (unless otherwise indicated). After the cells were allowed to attach and grow for 48 hours, the cell culture medium was changed and different drug treatments were introduced at that time. In most experiments, the drug treatment lasted for 6 days with one medium change on the fourth day following the initial drug treatment.

Preparation of the Anticancer Drug Solutions

Due to their high lipophilicity, the stock solutions of 2-MeO-E_2 (10 mmol/L) and paclitaxel (0.2 mmol/L) were prepared in pure ethanol (200 proof). The stock solutions for doxorubincin (5 mmol/L), mitomycin C (0.1 mg/mL), and vinorelbine (1 mmol/L) were prepared in phosphate buffer (pH 7.4). The stock solution containing 10 mmol/L 5-fluorouracil was prepared by first making a 50 mmol/L drug concentration in 1.0 N potassium hydroxide and followed by dilution with phosphate buffer (pH 7.4) to a 10 mmol/L drug concentration. All these stock solutions were filtered with a Millex syringe filter (0.22 μm acetatecellulose membrane) and the filtrates were stored at −20°C in tightly sealed sterile tubes. Shortly before introducing the anticancer agents to the cultured cancer cells, each chemical was freshly diluted with a buffer to the desired concentrations and an aliquot (usually 10 μL) of the drug-containing solution was added to each well. Usually, <0.1% of the original solvent of the stock solution was present in the final cell culture medium.

Measurement of Cell Growth

The cell density in the 96-well microplates was determined by using the crystal violet staining method (12, 17). Briefly, the culture medium in the microplates was first removed by aspiration, and then the cells in each well were fixed with 1% glutaraldehyde for 15 minutes. After removing the fixation solution, each well was rinsed with PBS buffer and allowed to dry at room temperature. The cells in each well were then stained with 50 μL of 0.5% crystal violet (dissolved in 20% methanol and 80% deionized water) for 15 minutes at room temperature, and the plates were rinsed carefully with tap water to remove residual crystal violet. The stained dye was then dissolved in 100 μL of 0.5% Triton X-100 overnight. After addition of 50 μL of 200-proof ethanol, the absorbance values of each well were measured at 560 and 405 nm with a UV_{max} microplate reader (Molecular Device, Palo Alto, CA), and the difference in the absorbance values at these two wavelengths were used to represent the cell density.

Metabolic Interconversions between E_2 and E_1 in Intact Human Breast Cancer Cells

Confluent cells growing in the 96-well plates were used to determine the rate of metabolic interconversion between E_2 and E_1. Cells were incubated with 100 μL serum-free culture medium (supplemented with 100 IU/mL penicillin and 100 μg/mL streptomycin) and desired concentrations of E_2 or E_1 (containing ~0.2 μCi of ^{3}H-labeled E_2 or E_1). At different time points of the incubation, the medium in each culture well was removed and transferred to a microcentrifuge tube and 50 μL of methanol was added to each tube. After a brief vortex and centrifugation for 10 minutes at 14,000 rpm, an aliquot (50 μL) was injected into HPLC for analysis of the radioactive composition of E_2, E_1, and their metabolites.

The HPLC system used for this purpose consisted of a Waters 2690 separation module, a β-RAM radioactivity detector (IN/US Systems, Inc., Tampa, FL), a Waters UV detector (model 484), and a Ultracarb-5 octadecyl silane column (150 × 4.60 mm, Phenomenex, Torrance, CA). The solvent system for the separation of E_2, E_1, and their metabolites consisted of acetonitrile (solvent A), water (solvent B), and methanol (solvent C); the solvent gradient (A/B/C) was as follows: 3 minutes of isocratic at 16:68:16, 0.5 minutes of gradient to 45:45:10, 10 minutes of isocratic at 45:45:10, and then 0.5 minutes of gradient to 16:68:16. The gradient was then held for 6 minutes before analysis of the next sample. The quantification of the ^{3}H-labeled E_2, E_1, and its metabolites was based on radioactivity measurements, and their identification was based on their retention times on the HPLC. Gas chromatography–mass spectrometry (GC-MS) analysis of the metabolically formed 2-methoxy-E_1 was used for the unequivocal identification of its structure.

Enzymatic Interconversion between E_2 and E_1 and between 2-MeO-E_2 and 2-Methoxy-E_1

For kinetic analysis of the enzyme-mediated interconversion between the 17β-hydroxyl form of an estrogen and its 17-keto form, whole cell homogenates were used. To prepare whole cell homogenates, large-scale culture of each cell line was first made, and the harvested cells were then sonicated and homogenized in 50 mmol/L sodium phosphate buffer (pH 7.4, containing 1 mmol/L EDTA). The whole cell homogenates were stored at −80°C in 200 μL aliquots, and the protein content of the homogenates was determined by using the Bio-Rad protein assay with BSA as standard.

Enzymatic reactions consisted of 0.5 mg/mL protein of the whole cell homogenates, desired concentrations of the nonradiolabeled substrate

(containing 0.2-0.5 μCi of ^{3}H-labeled estrogen substrate), 2 mmol/L of a supporting cofactor in a final volume of 200 μL of 50 mmol/L sodium phosphate buffer [containing 1 mmol/L EDTA (pH 7.4)]. The incubation was carried out at 37°C for 1 hour (unless otherwise indicated). The amount of the radioactive estrogen metabolites formed and the amount of the substrates remained after incubation were determined using our HPLC analytic method as aforementioned.

Reverse Transcription-PCR Analysis of the Expression of the Oxidative 17β-HSD Isozymes and the Human UDPGT$_h$-2 in Cultured Cells

Primers. Three known oxidative 17β HSD human isozymes (namely, types II, IV, and VIII) were analyzed in the present study. For type II 17β-HSD, the forward primer was designed as 5-GCT GGT CTT GGT ATT TGC-3, which corresponds to its mRNA sequence 479 to 499, and the backward primer was 5-CTT GTC ACT GGT GCC TGC GAT-3, complementary with its mRNA sequence 972 to 912 (namely, 514 bases of the amplified sequence). For type IV 17β-HSD, the forward primer was 5-GCT CTG GAG GCT TTG GTG GAA-3, corresponding to its mRNA sequence 1,442 to 1,462, and the backward primer was 5-GGC GGC GTC CTA TTT CCT CAA-3, complementary with its mRNA sequence 1,931 to 1,951 (namely, 510 bases of the amplified sequence). For type VIII 17β-HSD, the forward primer was 5-TCT CGC CCA CCA TCT GTC GTT-3, corresponding to its mRNA sequence 307 to 327, and the backward primer was 5-CCA AGA ATG CGA CCA CAT CTG CC-3, complementary with its mRNA sequence 726 to 748 (namely, 442 bases of the amplified sequence).

The forward primer for human UDPGT$_h$-2 (also called type IV UDPGT) was designed as 5-ACC TGC CAA ACC CCT GCC TAA G-3, corresponding to its mRNA sequence 863 to 884, and the backward primer was 5-CAC ACA GAC CAG CAG GAA CCC AA-3, complementary with its mRNA sequence 1,495 to 1,517. The amplified sequence is a 655 base fragment within the translated region.

Reverse transcription-PCR. For the isolation of the total RNAs, 1 mL Tri reagent (containing guanidine isothiocanide, phenol, and sarkosyl) was added to the cultured cells. After repetitive pipetting to dissolve the cells, the mixture was transferred to a 1.5 mL Eppendoff tube and followed by addition of 200 μL chloroform, and then the mixture was vortexed until it appeared milky. The mixture was centrifuged at 14,000 rpm for 20 minutes. The supernatant was transferred to a new tube containing 500 μL isopropanol. After gentle reversing twice to thrice, the tube was centrifuged at 14,000 rpm for another 20 minutes. After removal of the supernatant, the pellet was washed with 75% ethanol thrice and then it was dissolved in TE buffer.

For RT-PCR, 5 μg of the total RNA in 8 μL water was mixed with 2 μL oligo-dT and 2 μL deoxynucleotide triphosphate (dNTP) in a 1.5 mL Eppendoff tube. After incubation at 65°C for 15 minutes, the tube was chilled on ice. Each tube was then supplemented with 4 μL of the buffer, 1 μL RNase-out, and 2 μL DTT. After preheating at 37°C for 2 minutes, 1 μL reverse transcriptase was added. The tube was incubated at 37°C for 60 minutes. The reaction was terminated by incubation at 70°C for 15 minutes.

Statistical Analysis

In the *in vitro* growth inhibition experiments, the cell growth rate of the control and drug-treated groups were expressed as mean ± SE of the values obtained from six to eight replicate wells. The IC_{50} values were calculated according to the equation for the sigmoidal dose-response curves (with variable slopes) using the nonlinear regression curve-fitting model of the Prism software. Unless otherwise indicated, one-way ANOVA was used for multiple comparisons and the Tukey's test was used for the pairwise comparisons. The Michaelis-Menten curves and the Eadie-Hofstee plots for the enzyme kinetics were drawn by using the Prism software. $P < 0.05$ was considered to be statistically significant and $P < 0.01$ was considered statistically very significant.

Results

Selective Insensitivity of ZR-75-1 Cells to the Antiproliferative Effect of 2-MeO-E$_2$

The antiproliferative effect of 2-MeO-E_2 in the ER-negative MDA-MB-435s human breast cancer cells is shown in Fig. 2*A*. 2-MeO-E_2 inhibited the growth of these cells in a concentration-dependent manner and the average IC_{50} value was 0.8 μmol/L. The data comparing the antiproliferative effect of 2-MeO-E_2 in three ER-positive human breast cancer cell lines, MCF-7, T-47D, and ZR-75-1, are shown in Fig. 2*B-D*. Different cell lines had very different sensitivities to the antiproliferative action of 2-MeO-E_2. 2-MeO-E_2 inhibited the growth of MCF-7 cells in a concentration-dependent manner, with IC_{50} of 0.75 to 1.0 μmol/L, and it also inhibited the growth of T-47D cells with IC_{50} of 1.2 μmol/L. A near-complete inhibition of the growth of these two cell lines was reached when 2 μmol/L 2-MeO-E_2 was present. In contrast, increasing the concentrations of 2-MeO-E_2 in the culture medium from 0.1 to 2 μmol/L only exerted a very weak antiproliferative effect in the ZR-75-1 cells (Fig. 2*D*). Notably, the growth inhibitory effect in the ZR-75-1 cells was dependent on the density of the cells present in each culture well. When the ZR-75-1 cells were initially seeded in the 96 wells at a higher concentration (twice as usual, i.e., at 4×10^4 cells/well), the insensitivity of the cells to the antiproliferative actions of 2-MeO-E_2 became more pronounced.

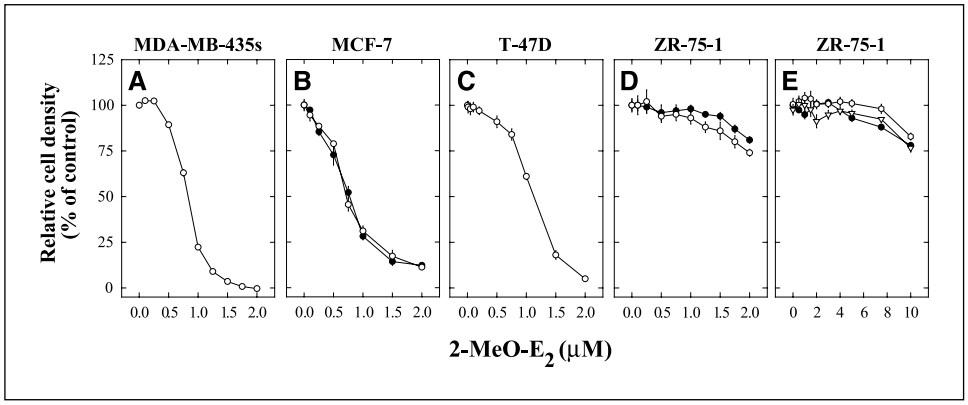

Figure 2. Comparison of the antiproliferative actions of 2-MeO-E_2 in four human breast cancer cell lines. The method for culturing the ER-negative MDA-MB-435 cells and the ER-positive MCF-7, T-47D, and ZR-75-1 cells was described in Materials and Methods. Experimental data in (*A*) to (*D*) were obtained when the cells were seeded at the initial density of 2×10^4 cells per well, but the data in (*E*) were obtained when the ZR-75-1 cells were seeded at a higher initial density (4×10^4 cells per well). Each cell line was treated with increasing concentrations of 2-MeO-E_2 for a total of 6 days. The cell density at the end of the 6-day treatment was determined by using the crystal violet staining method. *Points,* mean of five to six replicate measurements; *bars,* SE.

In several separate experiments when the cells were seeded at this higher density, the maximal inhibition of the growth of the ZR-75-1 cells was <20% at up to a 10 μmol/L concentration of 2-MeO-E_2 (Fig. 2*E*). In one of the experiments where cells were seeded at a high density, the maximal inhibition of the ZR-75-1 cell growth was ~40% at a 20 μmol/L concentration (data not shown).

To provide clues as to whether the observed low sensitivity of the ZR-75-1 cells to 2-MeO-E_2 was due to a nonspecific resistance of these cells to multiple anticancer drugs or due to a selective resistance only to 2-MeO-E_2, we compared the antiproliferative actions of several commonly used anticancer agents, including 5-fluorouracil, doxorubicin, mitomycin C, colchicine, paclitaxel, vinorelbine, vinblastine, and vincristine in all these four human breast cancer cell lines. We found that the ZR-75-1 cells had a similar overall sensitivity as the other three human breast cancer cell lines to the antiproliferative actions of these agents (Table 1). These results clearly suggest that the marked insensitivity of the ZR-75-1 cells to 2-MeO-E_2 was selective only to this anticancer steroid, which likely was mediated by a 2-MeO-E_2–specific mechanism that is different from the mechanism(s) of the usual multidrug resistance. In addition, we also observed that the ER-positive ZR-75-1 cells had a considerably low sensitivity to the antiestrogenic action of ICI-182,780 (a pure ER antagonist) compared with the ER-positive MCF-7 and T-47D cells (Table 1).

Metabolic Interconversion between 17β-Hydroxysteroids and 17-Ketosteroids in Intact Human Breast Cancer Cells

To determine the underlying mechanism(s) for the observed selective insensitivity of ZR-75-1 cells to 2-MeO-E_2, we set out to explore the possibility that this insensitivity may be caused by the presence of high levels of steroid-selective metabolizing enzyme(s) in these cells, which provides rapid metabolic inactivation of 2-MeO-E_2. Our initial experiments showed that when [^{3}H]E_2 or [^{3}H]E_1 were introduced to each of the four cell lines in culture, the distinct difference in their metabolism was the drastically different rates of interconversion between E_2 and E_1. Following this observation, we conducted a detailed comparison of the rates of interconversion between E_2 and E_1 (and between 2-MeO-E_2 and 2-methoxy-E_1) in these four cell lines and some of the data are described below.

Conversion of E_2 to E_1 and 2-MeO-E_2 to 2-methoxy-E_1. To determine the rate of metabolic conversion of E_2 to E_1 in different human breast cancer cell lines, cells were first grown to near confluence in the 96-well plates. The cells were then incubated for a desired length of time in the presence of 100 μL serum-free culture medium supplemented with 1 μmol/L E_2 (containing ~0.5 μCi ^{3}H-labeled E_2). The profile of E_2 metabolites present in the culture medium was determined by HPLC analysis.

When the ER-positive MCF-7 and T-47D cells were cultured under these experimental conditions for up to 24 hours (Fig. 3*A* and *B*), little or no E_1 was detected in the culture medium, although a small fraction (20-30%) of [^{3}H]E_2 was found to be converted to other radioactive metabolites (mostly water-soluble conjugates) during this period. Similarly, little or no E_1 was detected as a metabolite 24 hours after introduction of 1 μmol/L [^{3}H]E_2 to the ER-negative MDA-MB-435s cells (Fig. 3*D*), but ~20% of the original [^{3}H]E_2 was metabolically converted to a water-soluble conjugate.

When the ZR-75-1 cells were tested, the disappearance of 1 μmol/L [^{3}H]E_2 in the culture medium occurred very rapidly and in a time-dependent manner (Fig. 3*C*). The apparent half-life ($T_{1/2}$) of E_2 in the culture medium was only ~2 hours, and the concentrations of E_2 remaining after 12 and 24 hours of incubation were only 8.0% and 7.6%, respectively, of its initially introduced concentration (Fig. 3*C*). Our HPLC analysis showed that the major metabolite formed from E_2 was E_1, which accounted for ~90% of all radioactive E_2 metabolites detected. Notably, the rate of E_1 formation increased in a manner that is almost exactly opposite to the rate of E_2 disappearance, suggesting that the conversion of E_2 to E_1 was a predominant pathway of E_2 metabolism in this cell line.

To determine whether the presence of different E_2 substrate concentrations had any effect on the overall patterns of the metabolic conversion of E_2 to E_1 in cultured ZR-75-1 cells, three different concentrations of E_2 covering a wide range (10-1,000 nmol/L) were tested (one representative data set was shown in Fig. 4, *top*). The percent conversion of E_2 to E_1 and the apparent half-lives ($T_{1/2}$) were found to be independent of the [^{3}H]E_2 substrate concentrations present, suggesting that the metabolic conversion of E_2 to E_1 in intact ZR-75-1 cells primarily followed the first-order kinetics, with an apparent $T_{1/2}$ of ~2 hours.

For comparison, we also determined the metabolism of [^{3}H]2-MeO-E_2 at two subtoxic concentrations (0.1 and 0.5 μmol/L) in cultured ZR-75-1 cells under the same experimental conditions. The radioactive [^{3}H]2-MeO-E_2 used in these experiments was biosynthetically prepared and purified with HPLC a day before its use in the experiments (described in Materials and Methods). The metabolic patterns for the conversion of [^{3}H]2-MeO-E_2 to 2-methoxy-E_1 was almost the same as the pattern for the conversion of E_2 to E_1. The apparent $T_{1/2}$ was ~2.5 hours (Fig. 4, *bottom*).

Conversion of E_1 to E_2. We also determined the rate of metabolic conversion of E_1 to E_2 in these cell lines under the same conditions. When the ER-positive MCF-7 and T-47D cells were incubated with a medium containing 1.0 μmol/L [^{3}H]E_1, these two cell lines showed a similar overall pattern for the conversion of E_1 to E_2, with ~50% of E_1 converted to E_2 in MCF-7 cells and ~65% converted in T-47D cells after 24 hours (Fig. 3*A* and *B′*). In comparison, when the ER-positive ZR-75-1 cells were cultured in a medium containing 1.0 μmol/L [^{3}H]E_1, the majority (~80%) of the E_1 was not metabolized and only <20% of the E_1 was converted to other non-E_2 metabolites after 24 hours (Fig. 3*C′*).

In the ER-negative MDA-MB-435s cells, the rate of conversion of 1.0 μmol/L [^{3}H]E_1 to E_2 was slow (Fig. 3*D′*). Notably, although E_1 disappeared in cultured MDA-MB-435s cells in a time-dependent

Table 1. Comparison of the *in vitro* growth inhibitory potencies (IC_{50} values) of several anticancer agents in four human breast cancer cell lines

Anticancer agent	MDA-MB-435s	MCF-7	T-47D	ZR-75-1
5-Fluorouracil (μmol/L)	4.3	2.6	0.9	0.3
Doxorubicin (nmol/L)	98	120	42	66
Mitomycin C (nmol/L)	36.5	8.6	13.3	34.2
Colchicine (nmol/L)	7.0	7.2	9.4	7.2
Vinorelbine (nmol/L)	2.9	3.2	7.5	5.0
Paclitaxel (nmol/L)	3.0	2.0	7.0	6.4
Vinblastine (nmol/L)	0.7	0.5	0.7	0.8
Vincristine (nmol/L)	1.1	1.3	0.8	1.9
ICI-182,780 (μmol/L)	Not studied	0.16	0.34	1.15

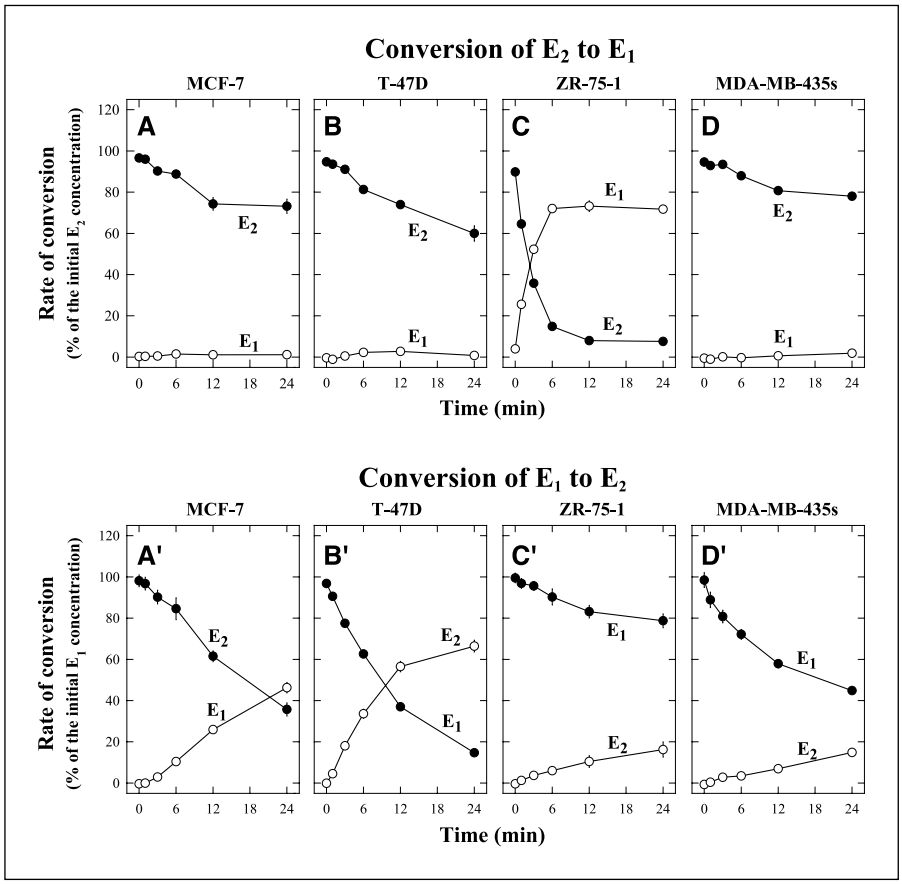

Figure 3. Rate of conversion of E_2 to E_1 (*top*) and conversion of E_1 to E_2 (*bottom*) in the ER-positive and ER-negative human breast cancer cell lines in culture. The methods for culturing the ER-positive MCF-7, T-47D, and ZR-75-1 cells and the ER-negative MDA-MB-435 cells were described in Materials and Methods. The concentration of [^{3}H]E_2 or [^{3}H]E_1 present in the medium was 20 nmol/L. *Points,* mean of triplicate determinations; *bars,* SE.

manner, most of the E_1 was converted to other metabolites (not E_2), including a glucuronidated metabolite of E_1 (based on its HPLC retention time).

In summary, for the oxidative conversion of the 17β-hydroxysteroids to 17-ketosteroids, the ZR-75-1 cells had the highest metabolic activity, whereas the other cell lines (MCF-7, T-47D, and MDA-MB-435s) had little or no detectable activity. For the reductive conversion of the 17-ketosteroids back to 17β-hydroxysteroids, the T-47D and MCF-7 cells had a high activity, whereas the ZR-75-1 and MDA-MB-435s cells had a very low activity. On the basis of these findings, it is apparent that the ZR-75-1 cells in culture have the highest net activity among the four cell lines tested that favors the metabolic accumulation of 2-methoxy-E_1, but not 2-MeO-E_2.

Reverse Transcription-PCR Analysis of the Expression of Oxidative 17β-HSD Isoforms in Human Breast Cancer Cells

The ZR-75-1 cells expressed type II 17β-HSD, whereas the MCF-7, T-47D, and MDA-MB-435s cells had no detectable levels of expression of the type II 17β-HSD (Fig. 5). In comparison, all four cell lines expressed types IV and VIII 17β-HSD, and no significant difference in the amount of their RNA levels was observed (Fig. 5). The expression level was not affected by treatment of these cells with 10 nmol/L E_2 for 24 or 48 hours.

The human $UDPGT_h$-2 is a major form of the glucuronsyltransferase that has specific activity for the glucuronidation of estrogens and is expressed in human breast cancer cells (18). The expression of this representative conjugation enzyme was also measured in all four cell lines. Whereas all four cell lines were found to express detectable levels of $UDPGT_h$-2, the level of expression was highest in the MDA-MB-435 cells, and only very low levels of this conjugating enzyme were expressed in the three ER-positive cell lines (Fig. 5). Notably, the high level of expression of the $UDPGT_h$-2 in MDA-MB-435s cells perfectly agreed with our observation that a substantial portion of the radioactive estrogen substrate was converted in a time-dependent manner to a glucuronide in these cells.

Enzyme Kinetic Characteristics for the Conversion of E_2 to E_1 and 2-MeO-E_2 to 2-Methoxy-E_1

Using crude whole cell homogenates prepared from cultured ZR-75-1 cells, we have also systematically characterized the biochemical properties of the 17β-HSD activity in this cell line. In addition, we have also compared the catalytic activity of 17β-HSD for the conversion of E_2 to E_1 and 2-MeO-E_2 to 2-methoxy-E_1 in all four cell lines. These biochemical studies were designed to validate our following suggestions: (*a*) the type II 17β-HSD in the ZR-75-1 cells is largely responsible for the rapid metabolic conversion of E_2 to E_1 and the conversion of 2-MeO-E_2 to 2-methoxy-E_1 in these cells and (*b*) the difference in the overall oxidative 17β-HSD activity in these four cell lines determines their differential sensitivity to 2-MeO-E_2. Some of the related data from this part of the study are summarized below.

Protein concentrations and incubation time. When 10 μmol/L E_2 was used as substrate and 2 mmol/L NAD as cofactor, the rate of conversion of E_2 to E_1 at different concentrations of cell homogenates (0, 0.25, 0.5, 0.75, and 1.0 mg protein per milliliter)

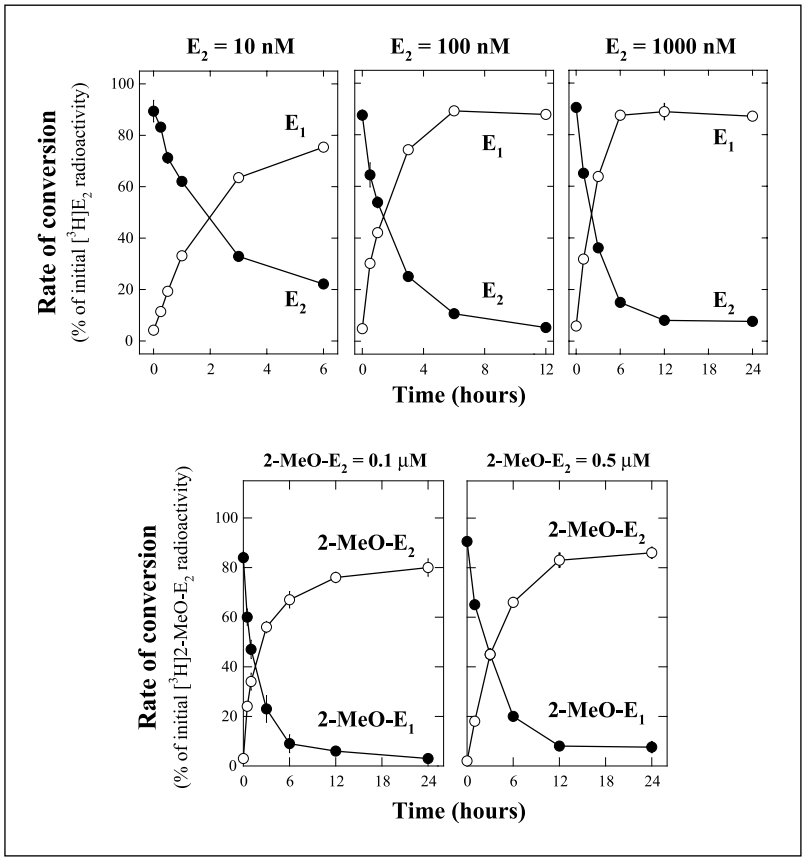

Figure 4. Rate of metabolic conversion of $[^3H]E_2$ to E_1 (*top*) or $[^3H]$2-MeO-E_2 to 2-methoxy-E_1 (*bottom*) in cultured ZR-75-1 cells when different concentrations of $[^3H]E_2$ (10, 100, or 1,000 nmol/L) or $[^3H]$2-MeO-E_2 (0.1 or 0.5 μmol/L) were present. The preparation of $[^3H]$2-MeO-E_2 was described in Materials and Methods. *Points,* mean of triplicate determinations; *bars,* SE.

correlated linearly with increasing protein concentrations (Fig. 6*A*). Under the same reaction conditions and at a homogenate concentration of 0.5 mg protein per milliliter, increasing the incubation time from 15 to 90 minutes resulted in an almost linear increase in the conversion of E_2 to E_1 (Fig. 6*B*). Similar patterns were also observed when 10 μmol/L E_1 was used as substrate and 2 mmol/L NADH as cofactor (Fig. 6*A′* and *B′*). Based on these assays, an optimized protein concentration of 0.5 mg/mL and an incubation time of 30 minutes were devised for most of the enzymatic assays.

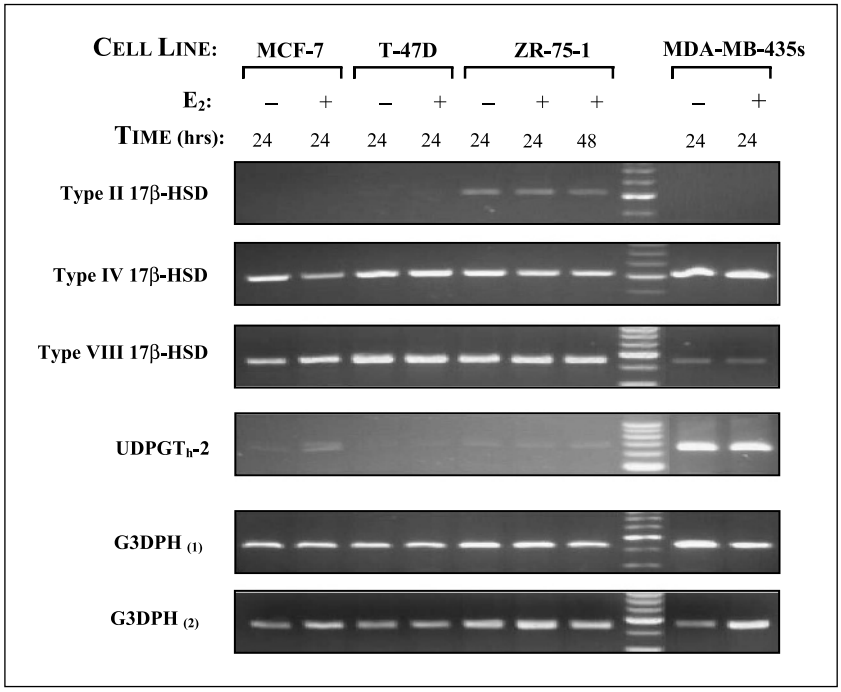

Figure 5. RT-PCR analysis of the expression of the oxidative human 17β-HSDs and $UDPGT_h$-2 in four human breast cancer cells. The primers used for the RT-PCR of each of the genes were described in Materials and Methods. Note that the data were collected from two separate experiments and the internal standard $G3DPH_{(I)}$ bands are for type II and IV 17β-HSDs and $UDPGT_h$-2, whereas the $G3DPH_{(II)}$ bands are for type VIII 17β-HSD. The multiple bands are the protein size markers.

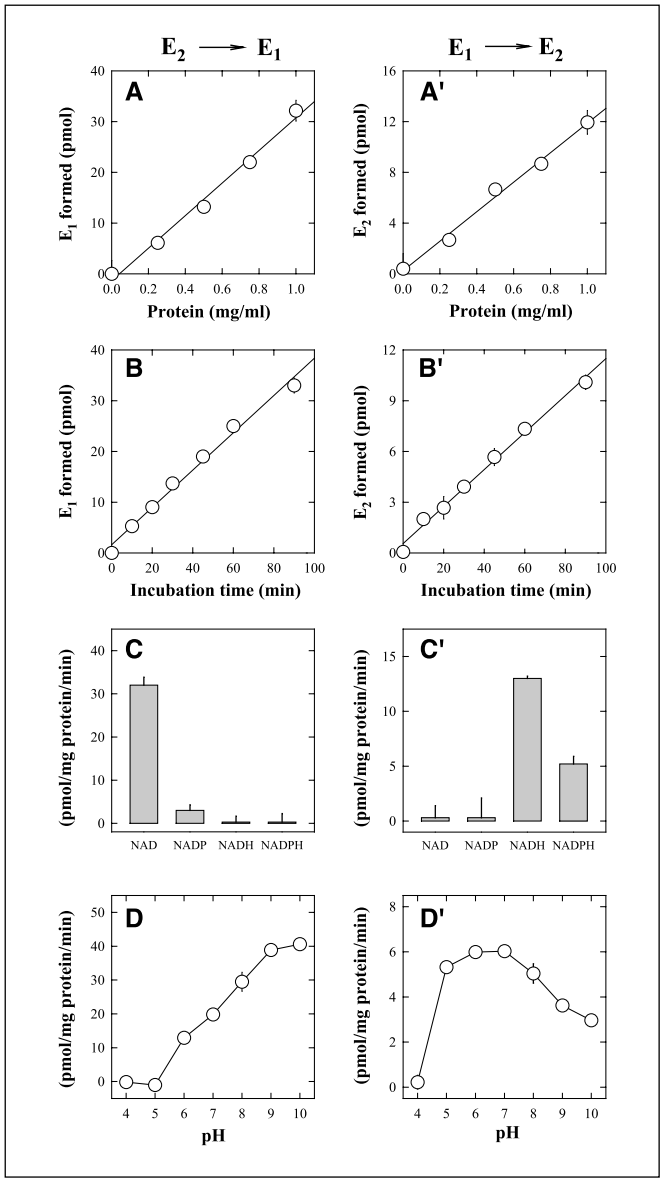

Figure 6. Effect of protein concentration, incubation time, cofactor (NAD, NADH, NADP, or NADPH), and reaction pH on the rate of interconversion between E_2 and E_1 catalyzed by the ZR-75-1 whole cell homogenates. The reaction conditions and the HPLC analysis of the radioactive estrogen metabolites formed are described in Materials and Methods. The substrate concentration used was 10 μmol/L. *Points/columns,* mean of triplicate determinations; *bars,* SE.

Cofactor preference. The ability of NAD, NADH, NADP, or NADPH to support the interconversion between E_2 and E_1 was compared when 10 μmol/L of [^{3}H]E_2 or [^{3}H]E_1 was incubated with ZR-75-1 whole cell homogenates (at 0.5 mg/mL protein) in the presence of each of the cofactors (at 2 mmol/L). For the conversion of E_2 to E_1, NAD was found to be the optimal cofactor and no appreciable conversion was detected when NADH, NADP, or NADPH was used as a cofactor (Fig. 6*C*). For the reverse conversion of E_1 to E_2, NADH was the best cofactor, followed by NADPH, but NAD and NADP could not support the reaction (Fig. 6*C′*).

Reaction pH. At pH 4 to 5, no detectable conversion of E_2 to E_1 was observed. The rate of conversion of E_2 to E_1 increased continuously from pH 5 to 9 roughly in a linear manner, and the conversion was nearly plateaued when the pH reached 9 (Fig. 6*D*). For the conversion of E_1 to E_2, no appreciable activity was detected at pH 4, but the rate increased rapidly when the pH increased from 4 to 5, and the rate remained high when the pH varied from 5 to 8. At pH > 8, the rate of conversion of E_1 to E_2 started to decrease (Fig. 6*D′*).

Incubation temperature. For the conversion of E_2 to E_1 (at pH 7.4), the enzymatic activity at 45°C was ~32% higher than that at 37°C, but this activity was completely destroyed when the temperature was increased to 60°C. At pH 9.0, the enzymatic activity was not significantly different when the temperature was increased from 37°C to 45°C, but again the activity was almost completely lost when the temperature was increased to 60°C (Fig. 7*A*).

For the conversion of E_1 to E_2, the enzymatic activity (at pH 7 and 9) was increased by 84% or 82%, respectively, when the incubation temperature was increased from 37°C to 45°C, but this activity was mostly lost at 60°C, regardless of the pH differences (Fig. 7*B*).

Inhibition by spiro-δ-lactone, 17α-estradiol, and progesterone. Spiro-δ-lactone is a potent, highly specific inhibitor of the human type II 17β-HSD (19, 20). The presence of spiro-δ-lactone in the reaction mixture caused a concentration-dependent inhibition of the enzymatic conversion of [^{3}H]E_2 to E_1 and also the conversion of [^{3}H]2-MeO-E_2 to 2-methoxy-E_1 (Fig. 7*A* and *B*), with highly similar IC_{50} values. It is of note that the IC_{50} values of spiro-δ-lactone measured in the present study were comparable with the earlier measurements when the recombinant human type II 17β-HSD was assayed as the enzyme for the conversion of E_2 to E_1 (19, 20). In addition, the presence of 17α-estradiol or progesterone at 2 or 10 μmol/L (two nonspecific inhibitors) also showed similar degrees of inhibition of the conversion of [^{3}H]E_2 to E_1 or [^{3}H]2-MeO-E_2 to 2-methoxy-E_1 (Fig. 7*C* and *D*).

Determination of enzyme kinetic parameters (K_m and V_{max}). The enzyme kinetics for the conversion of E_2 to E_1 were determined under the optimized *in vitro* reaction conditions. The incubation mixture consisted of 0.5 mg/mL of cell homogenate protein, 2 mmol/L of NAD, and different concentrations of E_2 in a final volume of 0.2 mL of 50 mmol/L potassium phosphate buffer [containing 1 mmol/L EDTA (pH 7.4)]. The Michaelis-Menten curve and the Eadie-Hofstee plot for the 17β-HSD–mediated conversion of E_2 to E_1 were shown in Fig. 8*A*. The apparent K_m and V_{max} values (calculated according to curve regression analysis) were 1.8 μmol/L and 37.0 pmol/mg protein/min, respectively.

Under exactly the same *in vitro* reaction conditions, we also determined the kinetic parameters for the conversion of radioactive 2-MeO-E_2 to 2-methoxy-E_1. The Michaelis-Menten curve and the Eadie-Hofstee plot were shown in Fig. 8*B*. The apparent K_m and V_{max} values (calculated according to curve regression) were 1.6 μmol/L and 35.3 pmol/mg protein/min, respectively. Notably, additional GC-MS analysis of the metabolite peak collected off the HPLC confirmed that the major metabolite formed from 2-MeO-E_2 was 2-methoxy-E_1 (data not shown).

Comparison of the reductive 17β-hydroxysteroid dehydrogenase activity in all four cell lines. The whole cell homogenates from the ZR-75-1 cells contained the highest catalytic activity for the conversion of E_2 to E_1 and 2-MeO-E_2 to 2-methoxy-E_1. In comparison, the activity in the MCF-7 and T-47D cells was very low, only one fifth of the activity in the ZR-75-1 cells, and homogenates from the MDA-MB-435s cells had the lowest levels of the reductive 17β-HSD activity, approximately one tenth of that in

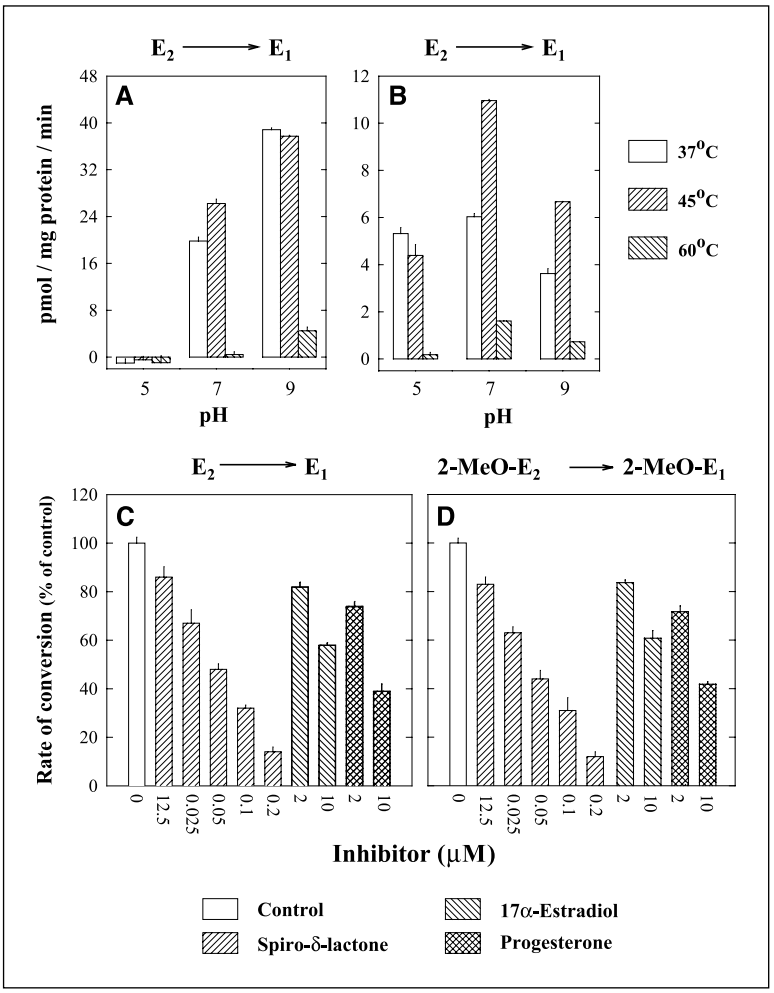

Figure 7. *A* and *B,* effect of different temperatures on the rate of interconversion between E_2 and E_1 catalyzed by ZR-75-1 whole cell homogenates (0.5 mg/mL). The substrate concentration used was 10 μmol/L. *Columns,* mean of triplicate determinations; *bars,* SE. *C* and *D,* inhibition of the conversion of E_2 to E_1 (*left*) or 2-MeO-E_2 to 2-methoxy-E_1 (*right*) by spiro-δ-lactone, 17α-estradiol, or progesterone by ZR-75-1 whole cell homogenates (0.5 mg/mL). The estrogen substrate concentration was 1 μmol/L, and NAD (2 mmol/L) was used as the cofactor. *Columns,* mean of triplicate determinations; *bars,* SE.

the ZR-75-1 cells (Fig. 8*C*). Notably, because RT-PCR analysis showed that the three sensitive cell lines (MCF-7, T-47D, and MAD-MB-435s) expressed no detectable levels of type II 17β-HSD mRNA (Fig. 5), the very low catalytic activity detected in the homogenates of these cell lines is likely contributed by other 17β-HSD isozymes (such as types IV and VIII).

Discussion

The results of our present study showed that the ER-positive ZR-75-1 human breast cancer cells were insensitive to the antiproliferative actions of 2-MeO-E_2. After comparing the sensitivity of the four cell lines used in this study to several other anticancer agents, including 5-fluorouracil, mitomycin C, doxorubicin, colchicine, vinorelbine, and paclitaxel, we noted that the ZR-75-1 cells were selectively insensitive to the anticancer actions of 2-MeO-E_2, which was not due to the development of a general multiple drug resistance in this cancer cell line. Also, we noted that the ER-positive ZR-75-1 cells had a lower sensitivity to the antiproliferative actions of ICI-182,780 (a pure ER antagonist) compared with the ER-positive MCF-7 and T-47D cells. Although the mechanisms for the antiproliferative actions of 2-MeO-E_2 and ICI-182,780 are completely different, both of these agents share the same steroid core structure with a free 17β-hydroxyl group. This similarity has led us to speculate that the selective insensitivity of the ZR-75-1 cells to 2-MeO-E_2 is likely attributable to a mechanism that is associated with the steroid chemical structure, such as due to the presence of high levels of certain steroid-specific metabolizing enzyme(s).

To probe the underlying mechanism(s) for this selective insensitivity, we compared all four cell lines for their ability to metabolize E_2 and 2-MeO-E_2. We found that when E_2 or 2-MeO-E_2 was added to the culture medium (at up to 1 μmol/L), they were almost completely converted to E_1 or 2-methoxy-E_1 in cultured ZR-75-1 cells within the first ~24 hours, whereas little conversion was detected in the sensitive MCF-7, T-47D, and MDA-MB-435s cells. Similarly, when the whole cell homogenates prepared from these four cell lines were assayed *in vitro* for the enzymatic activity to convert 2-MeO-E_2 to 2-methoxy-E_1 (or E_2 to E_1), we confirmed that the ZR-75-1 cells had the highest overall catalytic activity, whereas the activity in other three cell lines was much lower. Therefore, the ability of each cell line to convert 2-MeO-E_2 to 2-methoxy-E_1 (a metabolite with little or no anticancer activity) was, to a large extent, directly correlated with the insensitivity to 2-MeO-E_2.

The 17β-HSDs is a family of NAD(H)- and/or NADP(H)-dependent enzymes that catalyze the interconversion between 17β-hydroxysteroids and 17-ketosteroids (21–26). These enzymes have a widespread distribution in gonadal and extragonadal tissues. At least eight isozymes of 17β-HSD (types I-VIII) have already been identified (21–26). Each of these isozymes generally is classified either as oxidative or reductive 17β-HSD on the basis

of the dominant reaction it catalyzes. Whereas the oxidative isozymes (including types II, IV, and VIII) catalyze the conversion of 17β-hydroxysteroids to 17-ketosteroids, the reductive isozymes (types I, III, V, VI, and VII) predominantly catalyze the reduction of 17-ketosteroids to 17β-hydroxysteroids.

Our data on the interconversion between E_2 and E_1 in cultured MCF-7 and T-47D cells were largely in agreement with those reported earlier (27, 28). Notably, a few earlier studies have suggested that the oxidative activity of 17β-HSD present in ZR-75-1 cells was due to the presence of the type II isozyme (25, 29–31), a 42.8 kDa enzyme (mRNA = 1.4 kb) consisting of 387 amino acid residues (21, 32, 33). Using RT-PCR, we determined the mRNA levels of various oxidative 17β-HSD isozymes (namely, types II, IV, and VIII) in all four cell lines used in this study. The ZR-75-1 cells expressed high levels of type II 17β-HSD, whereas the MCF-7, T-47D, and MDA-MB-435s cells expressed no detectable levels of type II 17β-HSD (Fig. 5). Because our data showed that all four cell lines expressed similar levels of type IV and VIII 17β-HSD, and also because the overall reductive 17β-HSD activity in the whole cell homogenates from these three sensitive cell lines were much lower than the activity in ZR-75-1 cells (Fig. 8*C*), it is clear that the relative contribution of the types IV and VIII 17β-HSDs to the overall conversion of 2-MeO-E_2 to 2-methoxy-E_1 (or E_2 to E_1) is of far lesser importance compared with the type II 17β-HSD.

Providing further support to the suggestion that the type II 17β-HSD present in the ZR-75-1 cells is the dominant isozyme that is largely responsible for the conversion of 2-MeO-E_2 to 2-methoxy-E_1, we also showed that the kinetic characteristics of the 17β-HSD activity in the ZR-75-1 whole cell homogenates were highly similar to those of purified type II 17β-HSD in many ways. (*a*) The effect of pH (pH 7 and 9) on the two enzyme activities was similar. (*b*) The 17β-HSD from the ZR-75-1 cells was almost unchanged when the temperature was increased from 37°C to 45°C (at pH 9), but the activity was almost completely lost when it was further increased to 60°C. A very similar pattern in response to temperature change was also observed with the purified human type II 17β-HSD isozyme but not with other isozymes, such as types I and III (19). (*c*) NAD was the optimal cofactor for the enzymatic conversion of E_2 to E_1, whereas NADH was the optimal cofactor for the reversed conversion of E_1 to E_2. These cofactor requirements are the same as observed earlier with the purified human type II 17β-HSD (32). (*d*) The apparent K_m values for the 17β-HSD in the ZR-75-1 cells were similar to those determined for the purified type II 17β-HSD isozyme (20, 32, 33). Taken together, these data clearly suggest that the main oxidative activity of 17β-HSD present in the ZR-75-1 cells came from the type II isozyme.

In this study, we also found that the biochemical properties for the metabolic conversions of E_2 to E_1 and 2-MeO-E_2 to 2-methoxy-E_1 are almost identical: (*a*) very similar K_m and V_{max} values, (*b*) very similar response to temperature changes, and (*c*) very similar inhibition patterns by spiro-δ-lactone (a specific type II 17β-HSD inhibitor refs. 19, 20), 17α-estradiol, and progesterone. Collectively, these data showed that the same type II 17β-HSD that catalyzes the conversion of E_2 to E_1 in the ZR-75-1 cells also catalyzes the conversion of 2-MeO-E_2 to 2-methoxy-E_1.

It is of note that the type II 17β-HSD is broadly expressed in endometrial hyperplasia and carcinoma as well as certain forms of breast cancer (21, 33–35). In addition, a high activity of the oxidative 17β-HSD is present in the liver and kidney (21, 33). The high activity of the hepatic oxidative 17β-HSD may contribute importantly to the extensive metabolic inactivation when 2-MeO-E_2 is

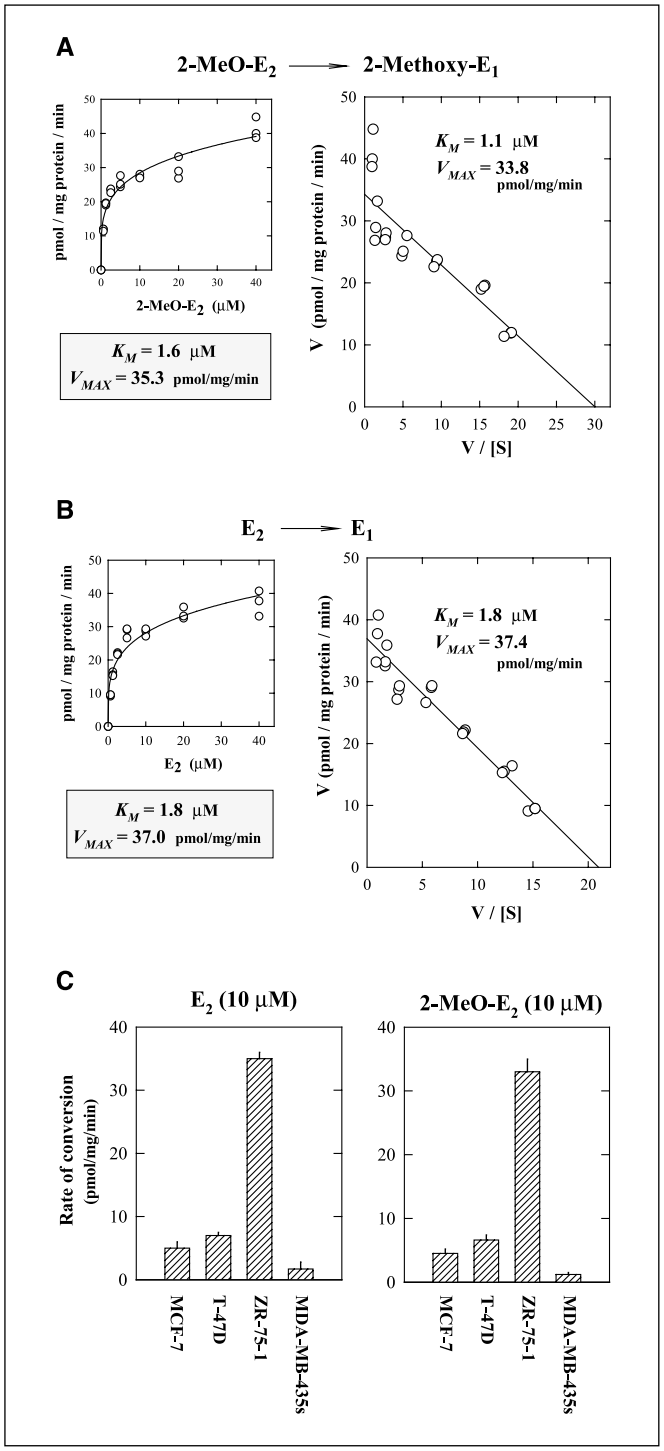

Figure 8. *A* and *B,* Michaelis-Menten kinetics of the conversion of E_2 to E_1 or the conversion of 2-MeO-E_2 to 2-methoxy-E_1 by the ZR-75-1 whole cell homogenates. The Eadie-Hofstee analysis of the data was done (*right*). Note that two sets of the kinetic parameters (K_m and V_{max}) were listed, which were separately calculated by using the curve-fitting functions of the SigmaPlot software or the Eadie-Hofstee analysis. *C,* the oxidative 17β-HSD activity for the conversion of E_2 to E_1 or 2-MeO-E_2 to 2-methoxy-E_1 by the whole cell homogenates from each human breast cancer cell line. The incubation mixture consisted of 0.5 mg/mL protein of the whole cell homogenates, E_2 or 2-MeO-E_2 (containing 0.2-0.5 μCi of ^{3}H-labeled substrate), and 2 mmol/L NAD in a final volume of 200 μL of 50 mmol/L sodium phosphate buffer [containing 1 mmol/L EDTA (pH 7.4)]. The incubation was carried out at 37°C for 1 hour. The radioactive E_1 or 2-methoxy-E_1 formed was determined by using HPLC analysis with radioactivity detection. *Columns,* mean of triplicate determinations; *bars,* SE.

given to patients through oral administration for treatment of the cancer. In line with this suggestion, 2-methoxy-E_1 (not 2-MeO-E_2) was found to be the major metabolite present in the circulation of pregnant women (2). In addition, recent pharmacokinetic studies in humans and animals also showed that large amounts of 2-methoxy-E_1 (instead of 2-MeO-E_2) were present in circulation after oral administration of 2-MeO-E_2. Because 2-methoxy-E_1 has little antiproliferative activity compared with 2-MeO-E_2 in cultured breast cancer cells (data not shown), the presence of high levels of the oxidative 17β-HSD that catalyze rapid conversion of 2-MeO-E_2 to 2-methoxy-E_1 is believed to significantly reduce the sensitivity of cancer cells to 2-MeO-E_2. In this context, it is of note that novel derivatives of 2-MeO-E_2 with modifications at the 17β position to make them less susceptible to type II 17β-HSD–mediated metabolic inactivation may show greater anticancer activity. Similarly, the results of our study also suggest that the use of selective type II 17β-HSD inhibitors may effectively enhance the anticancer activity of 2-MeO-E_2.

In summary, the results of our present study showed that the ZR-75-1 human breast cancer cells are selectively insensitive to the antiproliferative actions of 2-MeO-E_2. Mechanistically, the insensitivity to 2-MeO-E_2 is associated with the rapid metabolic conversion of 2-MeO-E_2 to 2-methoxy-E_1. Overall, our enzyme kinetic studies of the conversion of 2-MeO-E_2 to 2-methoxy-E_1 and E_2 to E_1 along with our studies of the 17β-HSD gene expression clearly shown that the type II 17β-HSD is predominantly responsible for the oxidative inactivation of 2-MeO-E_2. Our results also suggest that derivatives of 2-MeO-E_2 with modifications at the 17β position to have a reduced susceptibility to the 17β-HSD may have stronger anticancer activity, and a concomitant use of a potent selective 17β-HSD inhibitor may enhance the anticancer activity of 2-MeO-E_2 in breast cancer patients.

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