Epidemiology studies have identified several risk factors for breast cancer in women; these include early age at menarche, parity, late age at menopause, and late age at first pregnancy [reviewed in (1,2)]. All of these conditions are associated with increased lifetime exposure to estrogens, which is an important risk factor for this disease. The precise role of 17β-estradiol (E₂) in development of mammary cancer is unknown; however, it is possible that this hormone or its metabolites may influence more than one step in the complex pathways that lead to tumor formation (3–7).

It has been suggested that metabolism of E₂ into 2- and 16α-hydroxy metabolites, such as 2-hydroxyestrone (2-OHE1) and 16α-OHE1, may play a role in mammary carcinogenesis and that 2-OHE1 and 16α-OHE1 may serve as biomarkers for this process (8–10). Several in vivo and in vitro studies have demonstrated that altered metabolism of E₂ to give increased 16α- and lower 2-hydroxy metabolites is correlated with event(s) associated with tumor development (8–15). For example, metabolism of E₂ to 16α-OHE1 in mammary terminal duct lobular units, a target for mammary carcinogens, was higher in breast cancer patients versus controls (13,14). These data were consistent with laboratory animal studies in mice with different susceptibilities for development of mammary tumors (11), and it was reported that the 16α-/2-OHE1 metabolite ratio was higher in breast cancer patients versus controls (15).

It was also reported that the 16α-/2-OHE1 metabolite ratio in MCF-7 cells treated with various compounds was a predictor of mammary carcinogenesis (8). For example, 10⁻⁵ M concentrations of various pesticides and the mammmary carcinogen 7,12-dimethylbenz[a]anthracene (DMBA) increased this ratio, whereas the anticarcinogen indole-3-carbinol (13C) and omega-3 fatty acids decreased the ratio in MCF-7 cells (8). These results were surprising for two reasons. 13C induced CYP1A1 at concentrations greater than 10⁻⁴ M (16–18); therefore, it was surprising that CYP1A1-dependent E₂-2-hydroxylase activity was induced by 10⁻⁵ M 13C. Also, most of the organochlorine compounds used in the prior study enhance CYP2B gene expression, and this response is not usually induced in cancer cell lines (19). Results of initial studies on induction of E₂-2-hydroxylase activity in MCF-7 cells by pesticides, mammmary carcinogens, and anticarcinogens gave results (20) that were both similar and in contrast to those previously reported (8). For example, 10⁻³ M 13C and DMBA, respectively, increased and decreased 2-hydroxylase of E₂ by using the radiometric assay as previously reported; however, the mammmary carcinogen benzo[a]pyrene (BaP) increased and the anticarcinogens ICI 164,384 and 182,780 decreased E₂-2-hydroxylase activity (20). It is also possible that variability between studies may also be related to the instability of this cell line (21,22). In this study, low passage MCF-7 cells were used and monitored for both their estrogen and aryl hydrocarbon responsiveness. This study reports the effects of pesticides, mammmary carcinogens, and anticarcinogens on E₂ 16α-hydroxylase activity and 16α-/2-OHE1 metabolite ratios in MCF-7 cells. After treatment of MCF-7 cells with 10⁻⁵ M concentrations of the test compounds, the 16α-/2-OHE1 metabolite ratios in MCF-7 cells did not predict mammmary carcinogens.

**Materials and Methods**

**Chemicals.** [16α-³H]E₂ was custom synthesized by New England Nuclear (Boston, MA) with specific activity of 1.00 mCi/ml and a concentration of 27.9 pmol/ml, and was certified 99% pure by thin-layer chromatography (TLC) and HPLC. [2-³H]E₂ was purchased from New England Nuclear with a specific activity of 1.00 mCi/ml and a concentration of 45.45 pmol/ml. ICI 182,780 (7α-(9-(4,4,5,5,5-pentafluoropentylsulfinyl)nonyl)-1,3,5(10)triene-3,17-β-diol) was furnished by Alan Wakeling (Zeneca Pharmaceuticals, Macclesfield, UK). Minimum essential medium (MEM) was purchased from Gibco RBL (Grand Island, NY) and fetal bovine serum was purchased from Intergen Company (Purchase, NY). Tamoxifen, 4-hydroxytamoxifen, linoelic acid, all-trans-retinoic acid, and 9-cis-retinoic acid were obtained from Sigma (St. Louis, MO). 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) was greater than 98% pure and synthesized in this laboratory. Kepone, 1,1-dichloro-2-(p-chlorophenyl)-2-(o-chlorophenyl)ethene (o,p'-DDE), 1,1,1-trichloro-2-(p-chlorophenyl)-2-(o-chlorophenyl)ethane (o,p'-DDT), and 1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene (p,p'-DDE) were greater than 99% pure and provided by the U.S. EPA Repository.
(Research Triangle Park, NC). BaP and DMBA were purchased from Aldrich Chemical Co. (Milwaukee, WI) and were greater than 98% pure. Atrazine (97%) was purchased from ChemService (West Chester, PA) and 13C was obtained from Aldrich Chemical Company. All other chemicals and biochemicals were the highest purity available from commercial sources.

**Radiometric E₂, 16α-hydroxylase activity in MCF-7 cells.** MCF-7 human breast cancer cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were grown to 85% confluency in T-150 flasks in minimal essential media + 10% fetal bovine serum and seeded into T-25 flasks (Corning, Corning, NY) at a density of 1.0 × 10⁶ cells/plate. After allowing 24 hr for attachment, cells were maintained with fresh serum-free media for 24 hr. Cells were preincubated for 48 hr with test compounds in 5 ml of DMSO (0.1%) or 5 μl DMSO alone (control) in media containing 2.5% dextran-coated charcoal-stripped fetal bovine serum. Cells were treated for 48 hr with equal doses of the same test compounds and [16α-³H]E₂ at a final activity of 100 nCi/ml in fresh serum-stripped media. Aliquots (5.0 ml) of freshly prepared activated charcoal solution containing 0.25% activated charcoal and 0.0025% dextran in 0.1 M Tris-HCl (pH 8.0) buffer were centrifuged for 10 min at 1500 × g (4°C) in borosilicate test tubes and the supernatant was removed. Culture media (5.0 ml) obtained after incubation with test compounds + radiolabeled E₂ were added to charcoal pellets, incubated for 30 min (4°C) with gentle vortexing, and then centrifuged for 15 min at 1500 × g (4°C). Supernatant was analyzed for radioactivity in five aliquots (0.99 ml) by scintillation counting. [16α-³H]E₂ background radioactivity was caused by the slow spontaneous loss of tritium. The background levels were determined in parallel experiments by ethanol-fixing MCF-7 cells prior to the addition of DMSO + [16α-³H]E₂ for each experiment and were subtracted from the total radioactivity of each treatment to determine the net E₂ hydroxylase activity (20). The background was 0.65% of the total radioactivity and did not increase significantly during the course of these studies. The average net metabolism of control cells was 2% of the total [16α-³H]E₂ substrate added. For each treatment, four plates of cells were processed at the same time; results are presented as mean ± standard error (SE) and as a percent of metabolism or metabolite ratios in controls (DMSO treatment). The value for each plate is the average of the quantitation of five aliquots of its charcoal-washed media. Each experiment was repeated at least once on a separate occasion. The standard error for the 16α-2-OHE1 ratio was determined by propagating the error of the two individual measurements, and treatments were compared against controls using Student’s t-test.

**Radiometric E₂, 2-hydroxylase activity in MCF-7 cells.** To determine the E₂-2-hydroxylase activity, a protocol identical to the one above was utilized, using [2-³H]E₂ as the radiolabeled substrate, as previously described (20). 16α-Hydroxylase activity and 2-hydroxylase activity were analyzed with analysis of variance (ANOVA) and Scheffe’s test.

**Results**

The effects of various chemicals on E₂ 2-hydroxylase activity (radiometric assay) in MCF-7 cells were recently reported (20), and this study has expanded the number of chemicals to determine their effects on E₂ 2- and 16α-hydroxylase activities and their 16α-2-OHE1 metabolite ratios. Figure 1 illustrates the concentration-dependent induction of E₂ 2-hydroxylase activity by TCDD, and this paralleled previous studies on induction of CYP1A1-dependent activity (23–26). TCDD also induced E₂ 16α-hydroxylase activity at concentrations from 10⁻⁸ to 10⁻¹⁰ M. 16α-2-OHE1 metabolite ratios were concentration dependent and varied from 1.02 to 0.57. Treatment of MCF-7 cells with the mammary carcinogen DMBA (10⁻⁷–10⁻³ M) decreased both E₂ 2- and 16α-hydroxylase activities; however, the 16α-2-OHE1 metabolite ratios were significantly greater than 1.0 at the 10⁻⁷ and 10⁻⁵ M concentrations (Fig. 2). Induction of CYP1A1-dependent E₂ 2-hydroxylase activity was observed after treatment of MCF-7 cells with 10⁻⁵ M BaP. However, the 16α-2-OHE1 ratios were dependent on the concentration of BaP and varied from 0.08 to 1.17; these results...
differed markedly from the corresponding ratios obtained for DMBA.

The remaining results summarized in Figures 2 to 4 were obtained using compounds previously investigated by Bradlow and co-workers (8) as well as several drugs that exhibit antitumorogenic and/or antitumorigenic activity. The results obtained for the pesticides were highly variable: 16α-/2-OHE1 ratios for 10^-5 M ketone, atrazine, p,p’-DDE, o,p’-DDE, DDE, and β-hexachlorocyclohexane were 1.82 ± 0.060, 0.71 ± 0.027, 0.66 ± 0.030, 1.56 ± 0.089, 1.14 ± 0.059, and 0.69 ± 0.052, respectively. Metabolite ratios for 13C were greater than 1.0 as previously reported (20), whereas the value for linoleic acid was 0.74 ± 0.039. The 16α-/2-OHE1 ratios for the antiestrogens tamoxifen (10^-5 –10^-8 M) and 4-hydroxytamoxifen were greater than 1.0 (Fig. 3), whereas ratios for the 10^-4, 10^-7, and 10^-6 M concentrations of the antiestrogen ICI 182,780 were 1.59 ± 0.30, 1.74 ± 0.09, and 2.19 ± 0.21, respectively (Fig. 4). Compared to control cells, the effects of all-trans and 9-cis-retinoic acid on E2 2- and 16α-hydroxylase activities and their ratios were minimal (Fig. 4).

**Discussion**

Bradlow and co-workers (8) previously suggested that the effect of various pesticides on the 16α-/2-OHE1 metabolite ratios in MCF-7 cells was a biologic marker of breast cancer risk. These studies were carried out by treating MCF-7 cells for 48 hr with [3H]E2 substrates and 10^-5 M concentrations of the various compounds. Previous reports have demonstrated that E2 2-hydroxylase activity is a CYP1A1-dependent response in in vitro cancer cell lines (23–26), whereas CYP1A2 is the major catalyst for this enzyme activity in vivo (27–29). CYP1A1 and CYP1A2 are important phase I drug-metabolizing enzymes, which are induced by various xenobiotics that bind the aryl hydrocarbon receptor. Our initial interest in reexamining this assay and its predictive utility stemmed from two observations: 1) it was reported that 10^-5 M 13C induced E2 2-hydroxylase activity (8), whereas induction of CYP1A1 by 13C in MCF-7 cells requires concentrations greater than 100 μM (16,17); and 2) most of the organochlorine pesticides used in the study resemble phenobarbital as inducers of CYP2B-dependent activity, and this induction response is not observed in most cancer cell lines. Using the radiometric assay for E2 2-hydroxylase activity, the reported up regulation of this response by 13C was confirmed (20) and was also observed in the present study (Fig. 2). 13C increased E2 2-hydroxylase activity after treatment for 2 hr (30); however, the amount of metabolite formed was low. BaP also induced this response (20); this contrasted with the marked decrease of E2 2-hydroxylase activity in MCF-7 cells treated with DMBA (8,20). Previous studies on induction of E2 2-hydroxylase activity in this laboratory (20) showed some differences and similarities with data reported by Bradlow and co-workers (8). This study has reexamined induction of both E2 16α- and 2-hydroxylase activities and metabolite ratios after treatment of MCF-7 cells with diverse pesticides, mammmary carcinogens, and anti-carcinogens in MCF-7 cells.

TCDD was used as a positive control because it induces CYP1A1-dependent activities (including E2 2-hydroxylase) in MCF-7 cells and other aryl hydrocarbon responsive tissues (18,23–26,30,31). This was also paralleled by induction of E2 2-hydroxylase activity (Fig. 1). I3C (10^-5 M) also induced E2 2-hydroxylase activity (Fig. 2) as previously reported (8,20), even though induction of CYP1A1 mRNA or immunoreactive protein by this compound is observed at concentrations greater than 10^-4 M in MCF-7 cells (16,17). This induction response may be related to elevation of other CYP isozymes, and this requires further investigation.

The report which suggested that the 16α-/2-OHE1 metabolite ratios in MCF-7 cells could be used as a predictive biologic marker for mammmary carcinogens showed that the ratio was highest (5) for atrazine, p,p’-DDE, o,p’-DDE, and p,p’-DDE and was also increased for the mammmary carcinogen DMBA (8). In this study, elevated metabolite ratios were also observed for 10^-5 M o,p’-DDE (1.56 ± 0.09), M, 1.38 ± 0.06), and DMBA (2.47 ± 0.21), whereas these ratios were not significantly greater than 1 for the remaining compounds. The most striking difference in 16α-/2-OHE1 metabolite ratios was observed for the mammmary carcinogens DMBA and BaP (Fig. 2). In cells treated with DMBA, metabolite ratios were higher than 1 for all concentrations, whereas the ratios for 10^-6
and 10⁻⁵ M BaP were 1.17 ± 0.08 and 0.08 ± 0.009, respectively. The 16α-2/-OHE1 ratios obtained for 10⁻³ M BaP and DMBA were the lowest and highest, respectively, for all compounds used in this study. The ratio was also investigated for a series of antiestrogens that act through the estrogen receptor (ER) (tamoxifen, 4'-hydroxytamoxifen, and ICI 182,780) or via crosstalk with the ER signaling pathway (TCDD, trans-retinoic acid, and δ-tetrahydroretinoic acid). The 16α-2/-OHE1 ratios for most of these compounds were ≤1.0; however, values from 1.59 to 2.19 were observed for the antiestrogen ICI 182,780 (10⁻⁸–10⁻⁶ M), which is being developed for treatment for mammary cancer (32).

Thus, results from these studies show that mammary carcinogens and antiestrogens both increase and decrease 16α/-2-OHE1 metabolite ratios in MCF-7 cells, and the ratio was not a biologic marker of breast cancer risk. The reasons for differences between this study and the prior report (8) are unknown; however, MCF-7 cell lines can be highly variable with respect to wild-type and variant ER expression and E₂ responsiveness (21,22), as well as inducibility of CYP1A1-dependent activities (23). Retention of CYP1A1 inducibility was periodically monitored in this study by confirming induction of CYP1A1-dependent E₂-2-hydroxylase (Fig. 1) and ethoxyresorufin O-deethylase activities (data not shown) by TCDD. A recent study by Telang et al. (10) summarized the effects of DMBA and BaP on 16α/-2-OHE1 metabolite ratios in explant cultures of human mammary terminal duct lobular units and human mammary epithelial 184-B5 cells. They reported that both DMBA and BaP increased 16α- and decreased 2-hydroxylation of E₂; this was accompanied by increased 16α/-2-OHE1 metabolite ratios. Thus, in contrast to MCF-7 cells, BaP did not induce CYP1A1-dependent E₂-2-hydroxylase activity in these in vitro systems. It is possible that assays using explant cell cultures may be more useful than breast cancer cell lines for predicting some classes of mammary carcinogens. However, it is clear from results of this study that a diverse group of compounds should be used to test the predictive utility of an in vitro bioassay.

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