

## 2-Methoxyestradiol Is an Estrogen Receptor Agonist That Supports Tumor Growth in Murine Xenograft Models of Breast Cancer

Tara E. Sutherland,<sup>1</sup> Michael Schuliga,<sup>1</sup>  
Trudi Harris,<sup>1</sup> Bedrich L. Eckhardt,<sup>2</sup>  
Robin L. Anderson,<sup>2</sup> Lilly Quan,<sup>1</sup>  
and Alastair G. Stewart<sup>1</sup>

<sup>1</sup>Department of Pharmacology, University of Melbourne, Parkville, Victoria, Australia and <sup>2</sup>Cancer Biology Laboratory, Peter MacCallum Cancer Centre, East Melbourne, Victoria, Australia

### ABSTRACT

**Purpose:** 2-Methoxyestradiol (2MEO) is being developed as a novel antitumor agent based on its antiangiogenic activity, tumor cell cytotoxicity, and apparent lack of toxicity. However, pharmacologic concentrations of 2MEO bind to estrogen receptors (ER). We have therefore examined the ER activity of 2MEO.

**Experimental Design:** Estrogenic actions of 2MEO were evaluated by changes in gene expression of the ER-positive (MCF7) breast tumor cell line and, *in vivo*, estrogenicity was assessed in breast tumor xenograft models and by measuring endocrine responses in uterus and liver.

**Results:** In the ER-positive breast tumor cell line (MCF7), microarray experiments revealed that 269 of 279 changes in gene expression common to 2MEO and estradiol were prevented by the ER antagonist, ICI 182,780. Changes in the expression of selected genes and their sensitivity to inhibition by ICI 182,780 were confirmed by quantitative reverse transcription–PCR measurement. Activation of ER in MCF7 cells by 2MEO was further confirmed by stimulation of an estrogen response element–dependent reporter gene that was blocked by ICI 182,780 (1  $\mu$ mol/L). Doses of 2MEO (15–150 mg/kg) that had no antitumor efficacy in either *nu/nu* BALB/c or severe combined immunodeficient mice bearing ER-negative MDA-MB-435 tumors had uterotropic and hepatic estrogen-like actions. In female *nu/nu* BALB/c mice inoculated with the estrogen-dependent MCF7 tumor cells, 2MEO (50 mg/kg/d) supported tumor growth.

**Conclusions:** Tumor growth enhancement by 2MEO at doses generating serum levels (100–500 nmol/L) that have estrogenic activity suggests that a conservative approach to the further clinical evaluation of this agent should be adopted and that its evaluation in breast cancer is inappropriate.

### INTRODUCTION

Recent advances in the treatment of breast cancer include the introduction of the epidermal growth factor (EGF) receptor kinase inhibitors and the use of existing agents in better-optimized regimens. Chemoprevention of tumor recurrence using tamoxifen has now been extended by the subsequent use of aromatase inhibitors (1). Nevertheless, some breast cancers remain difficult to treat, serving as stimulus to the search for novel treatments.

The estradiol (E<sub>2</sub>) metabolite, 2-methoxyestradiol (2MEO), has been identified as a potential novel antitumor agent combining antiproliferative activity on a wide range of tumor cell types with antiangiogenic actions (2, 3). Combined treatment with agents that possess antiproliferative and antiangiogenic activity results in antitumor synergy and reduced likelihood of antitumor drug resistance (4). The development of 2MEO as an anticancer agent is based on its combined antitumor properties, its reported low affinity for estrogen receptors (ER), and lack of toxicity (5, 6).

Preclinical studies indicate that 2MEO decreases growth of several solid tumors and has potential in the treatment of multiple myeloma (7), in which angiogenesis has been implicated (8). 2MEO has antiangiogenic actions at doses that decrease the growth of murine melanoma (9) and of the human breast tumor cell, MDA-MB-435, in severe combined immunodeficient (SCID) mice (10). Doses of up to 75 mg/kg p.o. were well tolerated and reported to have been without estrogenic activity (9). 2MEO is reported to reduce tumor volume in different solid tumor models in mice including angiosarcoma (11), lung (12), pituitary (13), prostate (14), and melanoma (9), as well as pancreatic metastases (11). In addition, 2MEO alone and in combination with dexamethasone improves survival in experimental myeloma that is resistant to doxorubicin treatment (8). However, some laboratories have not detected reductions in tumor volume, despite using doses that have been reported to be active in other tumor studies including MDA-MB-435 human tumor xenograft in *nu/nu* mice (15), methyl-nitrosourea–induced mammary cancer in the Sprague-Dawley rat (16), and DSL6A pancreatic tumor in the Lewis rat (17).

2MEO has low oral bioavailability and a short half-life (2), prompting efforts to develop novel analogues with superior pharmacokinetic and pharmacodynamic profiles (15, 18–20). Notwithstanding the unfavorable pharmacokinetic profile, 2MEO is being evaluated clinically in phase I/II trials in breast and other tumors. Doses up to 1.2 g daily show neither efficacy

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**Note:** T. Sutherland and M. Schuliga contributed equally to this work.

**Requests for reprints:** Alastair G. Stewart, Department of Pharmacology, University of Melbourne, Parkville, Victoria 3010, Australia. Phone: 613-83445675; Fax 613-83440241; E-mail: astew@unimelb.edu.au.

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nor toxicity (21). Pharmacokinetic studies reveal maximum plasma concentrations of  $\sim 60$  nmol/L (22). *In vitro* data indicate a threshold concentration for antiproliferative actions of  $\sim 100$  nmol/L. Thus, the lack of activity (efficacy or toxicity) of 2MEO in phase I evaluation may be explained by maximum plasma concentrations that are subthreshold for either antitumor or estrogenic actions.

The mechanisms of action of 2MEO are not yet clear (23). Cell cycle arrest at G<sub>2</sub>-M due to impairment of spindle formation is associated with changes in tubulin dynamics (24, 25). 2MEO has micromolar affinity for the [<sup>3</sup>H]colchicine binding site on tubulin, with complex actions on tubulin dynamics distinct from those of colchicine or taxol (24, 26). 2MEO induces apoptosis at antiproliferative concentrations involving activation by 2MEO of p38<sup>mapk</sup>, c-jun-NH<sub>2</sub>-kinase and nuclear factor κB (27) upstream of p53-dependent mechanisms (28, 29). Activation of caspases and the ensuing apoptosis have been ascribed to increased free radical levels (30) due to inhibition of superoxide dismutase (31, 32), although this conclusion is controversial (33). Death receptor 5 up-regulation has also been implicated in the apoptotic effects of 2MEO (34). Although the mechanism(s) of action(s) of 2MEO requires further investigation, there is agreement that activity at ER is not involved in the antiproliferative or proapoptotic effects on tumor or other cell types (18, 35).

It is frequently asserted that 2MEO lacks significant affinity for ER and therefore is nonestrogenic. These claims seem to be based on initial studies examining the potential for endogenous levels of 2MEO to bind to ER at physiologic concentrations (36, 37). The affinity of 2MEO for ER ( $K_i \sim 100$ -300 nmol/L) has been established using radioligand binding assays on rat uterine cytosol, which is rich in ERα (18, 37) and similar affinity estimates have been obtained in MCF7 cells (35). Endogenous plasma levels of 2MEO are greatest in the last 3 weeks of pregnancy ( $\sim 10$  nmol/L) and otherwise are  $<0.1$  nmol/L (38). There is little likelihood that endogenous 2MEO has significant physiologic actions through binding to ER.

In contrast to physiologic levels, pharmacologically active levels of 2MEO are predicted to have significant levels of binding to ER, given the overlap in the concentration ranges for antiproliferative effects and ER binding. However, it is not known whether 2MEO has agonist or antagonist actions, nor is it established whether such actions will show tissue dependency, as is the case with selective ER modulators (39). The present study was designed to evaluate the relationship between antitumor efficacy and estrogenic actions of 2MEO in murine models of human tumor growth. We also sought to establish whether 2MEO acts as an ER agonist or antagonist using the ER-positive MCF7 breast tumor cell line.

## MATERIALS AND METHODS

**Cell Culture.** MDA-MB-435 cells were maintained in RPMI 1640 (JRH Biosciences, Lenexa, KS) containing 10% heat-inactivated FCS (JRH Biosciences). Cultures were supplemented with 2 mmol/L L-glutamine (JRH Biosciences), 1 mmol/L nonessential amino acids (Sigma Chemical Co., St. Louis, MO), 1 mmol/L sodium pyruvate (Sigma). The MCF7 cell line was maintained in DMEM (Life Technologies, Inc.,

Grand Island, NY) containing 2 mmol/L L-glutamine supplemented with 5% (v/v) FCS, 100 units/mL penicillin G, and 100 μg/mL streptomycin (CSL Biosciences Ltd., Parkville, Victoria Australia).

**Radioligand Binding.** Rat uterine cytosol was prepared as previously described (18) and incubated with 0.2 nmol/L [<sup>3</sup>H]E<sub>2</sub> in a 10 mmol/L Tris buffer (pH 7.4; 1.5 mmol/L EDTA, 10% w/v glycerol, 1 mmol/L phenylmethylsulfonyl fluoride) in the presence and absence of ICI 182,780 to define nonspecific binding. Increasing concentrations of either E<sub>2</sub> or 2MEO were added to cytosol containing 0.2 nmol/L [<sup>3</sup>H]E<sub>2</sub> to determine IC<sub>50</sub> values for displacement of [<sup>3</sup>H]E<sub>2</sub>.

**Real-time Reverse Transcription-PCR.** To quantify gene expression, RNA was extracted from cells in culture and reversed transcribed for analysis by real-time PCR using TaqMan chemistry. MCF7 cells maintained in six-well culture plates were allowed to grow to monolayer confluence at which time they were serum-deprived in phenol red-free medium for 24 hours. The cells were incubated in vehicle or ICI 182,780 (Tocris Cookson Inc., Ellisville, MO) 30 minutes before treatment with 3 μmol/L 2MEO (Sigma) or 100 nmol/L E<sub>2</sub> (Sigma). Cells were incubated for a further 4 hours before total RNA isolation was done using RNeasy Mini-kits (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions. RNA was reverse transcribed into cDNA, using random primers, with TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA) using the following thermal protocol: 25°C (10 minutes), 37°C (60 minutes), and 95°C (5 minutes). mRNA transcript levels of *XBP-1*, *CD38*, *prostaglandin E synthase (PTGES)*, and *cyclin D1 (CCND1)* were assayed by real-time PCR with predeveloped assays (Applied Biosystems) and Supermix-UDG (Invitrogen Corp., San Diego, CA) reagents using the ABI Prism 7900 HT sequence analyzer (Applied Biosystems). 18S was used to normalize other RNA values. Threshold (CT) values for each reaction for XBP-1, CD38, PTGES, CCND1, and 18s rRNA were determined using SDS 2.0 software (Applied Biosystems).

**cDNA Microarrays.** For microarray analysis, total RNA was used in the preparation of fluorescent dye-labeled cDNA by a two-step chemical coupling procedure. Total RNA from serum-deprived cells grown in 185-cm<sup>2</sup> tissue culture flasks treated with agents as described for real time reverse transcription-PCR (RT-PCR) was extracted using RNeasy Midi columns (Qiagen) according to the manufacturer's instructions. RNA (50 μg) was incubated with 4 μg oligo(dT) primer and 5 μL Lucidea scorecard mix in a final volume of 19.4 μL at 70°C for 10 minutes. mRNA was reversed transcribed by 400 units SuperScript II reverse transcriptase (Invitrogen) using a dinucleotide triphosphate mixture containing aminoallyl dUTP in the presence of Lucidea scorecard mix. Bound RNA template was removed by alkaline hydrolysis before the labeled cDNA was purified using Qiaquick PCR purification columns (Qiagen). Complementary DNA, incorporating residues of aminoallyl deoxyuridine, was coupled to either Cy3 or Cy5 amino reactive dyes (Amersham Life Science, Buckinghamshire, United Kingdom) while bound to the purification column. Cy3- and Cy5-labeled cDNA from control and treated groups, respectively, were eluted in distilled water, pooled, and further purified using a single PCR purification column.

The dye-labeled cDNA was prepared for hybridization by evaporating to dryness in the presence of a blocking mix containing tRNA, Cot-1 DNA, polydeoxyadenylate 50-mer oligonucleotide, herring sperm DNA, and Denhardt's solution. Samples were resuspended in  $3 \times$  SCC with 50% (v/v) formamide and heated to  $100^\circ\text{C}$  for 3 minutes. Samples were then chilled on ice before the addition of SDS (final 0.125% w/v). Samples were hybridized (overnight in a humidified chamber maintained at  $42^\circ\text{C}$ ) to cDNA microarrays comprising a human 10.5 kb clone set fabricated onto glass slides at the Peter MacCallum Cancer Centre Microarray Facility ([www.ccgpm.org](http://www.ccgpm.org)). Washed microarray slides were scanned using an Agilent Technologies microarray scanner system (G2565BA, Wilmington, DE). Feature extraction involving spot identification and background subtraction was done using Quantarray analysis software (Perkin-Elmer, Norwalk, CT). Imperfect features resulting from scratches, poor hybridization, bright pixel contaminants (i.e., SDS), and abnormalities were removed from analysis. Gene expression data analysis was done using Gene Spring 5 (Silicon Genetics, Redwood City, CA) software. Dye normalization to remove label bias effects was achieved by Lowess normalization to correct for differences in hybridization efficiency.

**Estrogen Response Element Search.** Match software (<http://www.gene-regulation.com/pub/programs/match/>) was used to search the promoters of selected genes for potential ER binding sites. Sequences for the promoter regions of genes (selected from those affected by 2MEO and  $\text{E}_2$ ) were obtained from the National Center for Biotechnology Information genomic database, selected with the aid of locus link (<http://www.ncbi.nlm.nih.gov/LocusLink/>). Each promoter region examined consisted of the 10,000 bases upstream from the end of exon 1. Match software uses mononucleotide weight matrices of transcription factors, including ER, obtained from the TRANSFAC data base (<http://transfac.gbf.de/TRANSFAC/>). The ER profile of the TRANSFAC high-quality ER matrix we used had the core similarity cutoff value set to 1. By setting the core similarity cutoff value to 1, the potential binding site has to include the sequence of the five most conserved bases within a matrix. In the case of an estrogen response element (ERE), this is either repeat of the inverted palindrome found in the consensus ERE, GGTCAnnnTGACC (40).

**Estrogen Response Element Reporter Assay.** MCF7 cells were seeded into 96-well plates at a density of  $1.2 \times 10^4$  cells per well and allowed to adhere overnight in complete medium. Transfection was achieved by adding to each well 0.5  $\mu\text{g}$  ERE-secreted alkaline phosphatase reporter construct (BD Biosciences Clontech, Palo Alto, CA) and 0.02  $\mu\text{g}$  pGL3 luciferase control vector (Promega Corp., Madison, WI), prepared with 2.5  $\mu\text{L}$  SuperFect transfection reagent (Qiagen) in sera- and antibiotic-free media. After 2.5 hours in the presence of serum, transfected cells were washed with PBS and replenished in DMEM medium in the absence of serum or phenol red. ICI 182,780 (1  $\mu\text{mol/L}$ ) was then added, whereas  $\text{E}_2$  (100 nmol/L) and 2MEO (100 nmol/L) were added 30 minutes later. Cells were maintained for a further 48 hours before medium was removed for sampling. The levels of secreted alkaline phosphatase in 15  $\mu\text{L}$  medium were determined by a chemiluminescent procedure using the Great EscAPe SEAP

reporter system (BD Biosciences Clontech). Firefly luciferase was used to monitor transfection efficiencies between wells. To determine firefly luciferase levels in transfected cells, wells were washed with PBS and cells lysed in Glo Lysis buffer (Promega). The lysate was assayed for luciferase using the Steady-Glo Luciferase assay system (Promega). Chemiluminescence for both secreted alkaline phosphatase and luciferase assays was measured in 96 white well/black frame isoplates (Perkin-Elmer) using a Packard Top Count NXT microplate scintillation and luminescence counter.

**Mice.** Female *nu/nu* BALB/c mice (8-14 weeks) and female SCID mice (10 weeks) were obtained from Animal Resources Centre (Perth, Australia) and housed in a pathogen-free environment for a period of 1 week before the commencement of experiments. All experiments were conducted according to protocols approved by the Animal Experimentation Ethics Committee of the University of Melbourne. Animals were killed by overdose of the inhalation anaesthetic, isoflurane, and serum samples collected by cardiac puncture.

**Tumor Growth Studies.** Female *nu/nu* BALB/c mice were anaesthetized with methoxyflurane and inoculated by injection of  $1 \times 10^6$  MDA-MB-435 cells in a 20- $\mu\text{L}$  1:1 ratio suspension of Matrigel (growth factor enriched; BD Biosciences, Bedford, MA)/PBS into the fourth mammary fat pad. 2MEO treatment commenced when tumors had become palpable (between days 11 and 16). 2MEO (15, 50, or 150 mg/kg) or vehicle (10% DMSO/90% peanut oil) was administered i.p. daily. An observer blind to treatment estimated tumor volumes by measuring perpendicular diameters of the tumor to calculate a volume using the formula: (shortest diameter)<sup>2</sup>  $\times$  longest diameter  $\times$  0.52 (10). Caliper-estimated tumor volumes and body weight measurements were routinely made during treatment. At the conclusion of the treatment period, animals were killed and tumors excised and weighed. Studies in female SCID mice followed a similar protocol. However,  $1 \times 10^6$  MDA-MB-435 cells in 100  $\mu\text{L}$  PBS suspension were inoculated s.c. into the right flank. Oral treatment with 75 mg/kg 2MEO or vehicle (0.5% carboxymethylcellulose, Sigma) commenced on day 12. The vehicle was chosen to replicate that used by Klauber et al. (10).

**MCF7 Tumor Growth.** Female *nu/nu* BALB/c mice were inoculated with  $1 \times 10^6$  MCF7 cells in a PBS/Matrigel suspension into the fourth mammary fat pad, following the protocol of the *nu/nu* BALB/c MDA-MB-435 studies. Treatment with 2MEO (50 mg/kg) or vehicle (10% DMSO/90% peanut oil) given i.p. was initiated 4 hours before tumor inoculation and daily thereafter for 16 days. Presence of tumors was assessed by an observer blind to treatment and palpable masses were confirmed postmortem in H&E-stained sections to be tumor tissue by the morphologic assessment of the presence of a mass of viable tumor cells.

**Determination of Levels of 2-Methoxyestradiol.** The stability of the 2MEO when incubated with MCF7 cells was investigated. Incubations were carried out under the conditions of the experiment characterizing 2MEO (3  $\mu\text{mol/L}$ )-induced changes in gene expression. The supernatant and cells were sampled at 4 or 24 hours after the addition of 2MEO and stored at  $-70^\circ\text{C}$ . 2MEO was extracted from cells and supernatant into 3 mL *tert*-butyl-methyl ether before being evaporated to dryness

and reconstituted in 150  $\mu$ L acetonitrile:water (1:1). Samples were further vortexed for 2 minutes and centrifuged at  $10,000 \times g$  for 20 minutes. An aliquot of the supernatant was resolved on a Shimadzu high-performance liquid chromatography (HPLC) system composed of a LC-10AT pump, SIL-10AD autosampler, SCL-10AVP system controller, and a RF-10XL fluorescence detector, fitted with a Phenomenex Ultramex 3 C18 column (3  $\mu$ m,  $100 \times 4.6$  mm). The mobile phase was isocratic 37.5% acetonitrile in 20 mmol/L ammonium acetate buffer (pH 4.0), with a flow rate of 0.8 mL/min. 2MEO was detected at excitation and emission wavelengths of 205 and 320 nm, respectively. 2MEO was resolved from  $E_2$  and 2-hydroxyestradiol (both detectable at the aforementioned wavelengths), using isocratic HPLC separation, and the amounts of 2MEO were compared with the amounts in samples of medium incubated with 2MEO (3  $\mu$ mol/L) under the same conditions in the absence of MCF7 cells. The level of 2MEO in sera was assayed by HPLC. Extracts were prepared by combining 100 to 300  $\mu$ L sera with 3 mL *tert*-butyl-methyl ether. Samples were vortexed for 2 minutes and then centrifuged at  $10,000 \times g$  for 20 minutes. Supernatants were transferred to clean tubes and evaporated to dryness using a vacuum centrifuge. Extracts were reconstituted and resolved by HPLC as described for cell culture media above. Recoveries were determined using an internal standard,  $16\alpha$ OH 2MEO.

**Statistical Analysis.** For microarray and RT-PCR analysis, changes in gene expression were analyzed using Student's paired *t* test. All other data were subjected to ANOVA with Bonferroni's post hoc test to identify individual differences. Comparisons were considered to be statistically significant when  $P < 0.05$ .

## RESULTS

**2-Methoxyestradiol Induces Estrogen Receptor-Dependent Gene Expression Changes in MCF7 Cells.** Our estimates of the ER-affinity values for 2MEO (104 nmol/L) and  $E_2$  (0.18 nmol/L) obtained on rat uterine cytosol (18) are similar to those of LaVallee et al. (35) obtained on purified recombinant ER $\alpha$  receptor protein (2MEO, 21 nmol/L;  $E_2$ , 0.04 nmol/L) and to those obtained in MCF7 cells (2MEO, 42 nmol/L;  $E_2$ , 0.39 nmol/L) (41). We chose to investigate the effects of 2MEO at 3  $\mu$ mol/L and  $E_2$  at 100 nmol/L because these concentrations saturate rat cytosolic ER (Fig. 1).

To compare gene expression induced by 2MEO with that induced by  $E_2$ , MCF7 cells were incubated for a period of 4 hours with 3  $\mu$ mol/L 2MEO or 100 nmol/L  $E_2$ . Microarray analysis revealed that the expression of 279 genes changed significantly ( $P < 0.05$ ) in MCF7 cells treated with either 2MEO or  $E_2$ . A high level of correlation between changes induced by 2MEO and those induced by  $E_2$  was observed ( $r^2 = 0.89$ ,  $n = 279$ ,  $P < 0.05$ ), but the regression slope of  $1.25 \pm 0.03$  suggested that  $E_2$  had a higher maximum effect than 2MEO (Fig. 2A). Incubation of MCF7 cells with the ER antagonist, ICI 182,780 (1  $\mu$ mol/L, added 30 minutes beforehand) prevented changes in expression of 269 of the 279 genes regulated by 2MEO and  $E_2$  (data not shown).

The increases in expression of *XBP-1*, *PTGES*, and *CCND1* elicited by 2MEO (3  $\mu$ mol/L) were prevented by preincubation with ICI 182,780 (1  $\mu$ mol/L), whereas the

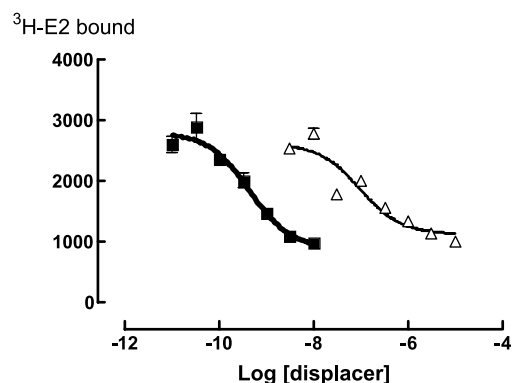


Fig. 1 Displacement of [ $^3$ H] $E_2$  from rat uterine cytosol by  $E_2$  (■) and 2MEO (△). Bars, range of the duplicate values where it exceeds the size of the symbol.

decrease in *CD38* expression was not prevented by the ER antagonist, confirming similar observations made with microarray analysis (Table 1).

The microarray and real-time RT-PCR data suggested that 2MEO has agonist actions at the ER. To ascertain whether 2MEO acts as an agonist on ER causing transactivation of ERE-containing promoters, we evaluated the actions of  $E_2$  and 2MEO on MCF7 cells transfected with an ERE reporter construct.  $E_2$  (100 nmol/L) and 2MEO (3  $\mu$ mol/L) each caused an increase in ERE activity, and the response to each stimulus was attenuated by preincubation in ICI 182,780 (1  $\mu$ mol/L), which itself caused a small but significant increase in ERE activity (Fig. 2B).

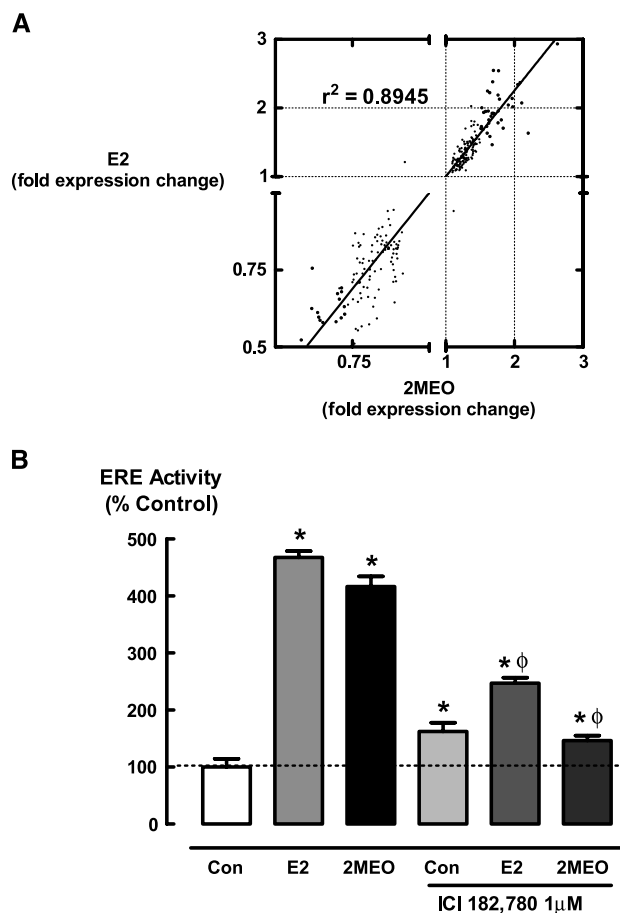
The stability of 2MEO was examined by incubation of 3  $\mu$ mol/L for 4 and 24 hours in MCF7 cell cultures. Resolution of the 2MEO on HPLC revealed a time-dependent decrease in 2MEO concentration without a concomitant increase in either  $E_2$  or 2-hydroxyestradiol. At 4 hours, the 2MEO level after incubation with cells was 64% and this declined to 22% after 24 hours (Fig. 3).

### Tumor Growth Is Not Reduced by 2-Methoxyestradiol.

We sought to establish a dose-response relationship for the antitumor and potential estrogenic actions of 2MEO in *nu/nu* BALB/c mice inoculated with  $10^6$  MDA-MB-435 cells (suspended in 50% Matrigel) by injection into the fat pad. Tumors were palpable 11 days after inoculation at which time the vehicle (10% DMSO/90% peanut oil, 100  $\mu$ L) or 15, 50, or 150 mg/kg 2MEO was given i.p. Progressive estimates of tumor volume failed to reveal any significant decreases in the rate of tumor growth in response to either 15 or 50 mg/kg 2MEO (Fig. 4). These findings were confirmed by postmortem tumor weight measurements. However, 2MEO, when given i.p. at 150 mg/kg, caused a significant increase in tumor growth that was confirmed by postmortem tumor weight measurement (Fig. 4).

2MEO had readily demonstrable effects on liver and uterine weights. Liver weight and uterine weight increased ( $P < 0.01$ ) following i.p. administration (Fig. 4), with no significant differences being observed between the effects of the 15-, 50-, or 150-mg/kg/d doses.

**2-Methoxyestradiol Has Estrogenic Actions and Lacks Antitumor Efficacy in Severe Combined Immunodeficient Mice.** Our initial failure to detect antitumor activity of 2MEO, despite exploring a wide dose range using similar



**Fig. 2** *A*, gene expression changes in response to incubation with E<sub>2</sub> (100 nmol/L) or 2MEO (3  $\mu$ mol/L) for 4 hours in MCF7 cells. RNA was isolated, reverse-transcribed, fluorescently labeled, and hybridized with microarrays of the 10.5 kb human gene set. The 279 genes that showed a significant difference to 1.0 ( $P < 0.05$ ) were plotted as means of fold changes in gene expression from at least four experiments.  $r^2$ , correlation between changes induced by E<sub>2</sub> and those induced by 2MEO. *B*, 2MEO (100 nmol/L) and E<sub>2</sub> (100 nmol/L) stimulate ERE activity in transiently transfected MCF7 cells compared with control (Con). Columns, mean ( $n = 6$ ); bars, SE. \*,  $P < 0.05$ , compared with control;  $\phi$ ,  $P < 0.05$ , compared with 2MEO or E<sub>2</sub> response.

conditions to those used in previous studies, prompted a more direct effort to replicate the conditions of the previous evaluation of 2MEO in breast tumor by Klauber et al. (10) in which SCID mice were inoculated s.c. with MDA-MB-435

cells and treated with 2MEO (75 mg/kg/d p.o.). The tumors were allowed to grow until palpable, at which time 2MEO (75 mg/kg) or vehicle (0.5% carboxymethylcellulose) was given p.o. daily for 18 days. 2MEO had no detectable effect on progressive tumor growth or postmortem tumor weight (Fig. 5). However, both liver weight and uterine weight significantly increased following p.o. administration.

**2-Methoxyestradiol Supports Estrogen-Dependent MCF7 Tumor Growth in *nu/nu* BALB/c Mice.** Our studies suggested that 2MEO reached concentrations *in vivo* that were able to activate ER because investigations of the liver and uterine enlargement in non-tumor-bearing female BALB/c mice indicated that these effects were partially inhibited by the ER antagonist, ICI 182,780 (data not shown). The MCF7 tumor cell line does not consistently engraft tumors unless mice are concurrently treated with E<sub>2</sub>. The ability of 2MEO to support the growth of MCF7 tumors was examined in *nu/nu* BALB/c mice. Treatment with 2MEO (50 mg/kg i.p. daily) from the day of inoculation resulted in the growth of histologically verified tumors (Fig. 6A) in 11 of 12 mice, whereas only 2 of 6 vehicle-treated mice grew MCF7 tumors and these appeared with a greater delay (Fig. 6B). Tumors were detected in 8 of 12 E<sub>2</sub> (2.5 mg/kg/d i.p.)-treated mice. The levels of 2MEO in serum samples peak concentrations of 100 to 400 nmol/L in the first 4 hours after treatment (Fig. 6C). These concentrations exceeded those required (10-100 nmol/L) for activation of the ERE when transfected into MCF7 cells (Fig. 2B) or for significant binding to ER (Fig. 1).

In separate experiments in normal non-tumor-bearing female BALB/c mice, administration of 2MEO (50 mg/kg/d i.p.) for 16 days also resulted in increases in liver and uterine weights (Table 2). In mice treated concurrently with ICI 182,780 (1 mg/kg/d i.p.) the increases in weight of liver was attenuated, but not blocked, whereas uterine weight did not show a significant increase (Table 2).

## DISCUSSION

2MEO has estrogenic actions at doses that are ineffective in regulating solid tumor growth as evidenced by three independent *in vivo* experiments. Moreover, *in vitro* studies provided unequivocal evidence for 2MEO acting as an estrogen agonist in MCF7 ER-positive tumor cells. Our findings and conclusions contrast with several studies suggesting that 2MEO does not have significant activity mediated through ER

**Table 1** Comparison of real-time RT-PCR and microarray determined changes in expression of *CCND1*, *CD38*, *XBP-1*, and *PTGES* in MCF7 cells incubated with 2MEO (3  $\mu$ mol/L), E<sub>2</sub> (100 nmol/L), or 2MEO and ICI 182,780 (1  $\mu$ mol/L) of four to seven observations

Gene	Analysis	2MEO		E <sub>2</sub>		2MEO + ICI	
		Mean $\pm$ SE	<i>P</i>	Mean $\pm$ SE	<i>P</i>	Mean $\pm$ SE	<i>P</i>
<i>CCND1</i>	Array	1.76 $\pm$ 0.09	0.001	1.96 $\pm$ 0.12	0.001	1.13 $\pm$ 0.10	0.280
	PCR	1.61 $\pm$ 0.22	0.049	2.12 $\pm$ 0.34	0.038	0.86 $\pm$ 0.29	0.643
<i>CD38</i>	Array	0.71 $\pm$ 0.06	0.007	0.69 $\pm$ 0.08	0.040	0.69 $\pm$ 0.02	0.003
	PCR	0.45 $\pm$ 0.10	0.001	0.77 $\pm$ 0.06	0.020	0.59 $\pm$ 0.19	0.098
<i>XBP-1</i>	Array	1.67 $\pm$ 0.14	0.008	1.92 $\pm$ 0.17	0.006	1.11 $\pm$ 0.06	0.162
	PCR	2.34 $\pm$ 0.40	0.016	3.14 $\pm$ 0.51	0.008	0.28 $\pm$ 0.07	0.002
<i>PTGES</i>	Array	2.63 $\pm$ 0.23	0.002	2.93 $\pm$ 0.46	0.013	1.08 $\pm$ 0.12	0.532
	PCR	2.91 $\pm$ 0.34	0.002	3.89 $\pm$ 0.86	0.043	0.54 $\pm$ 0.23	0.182

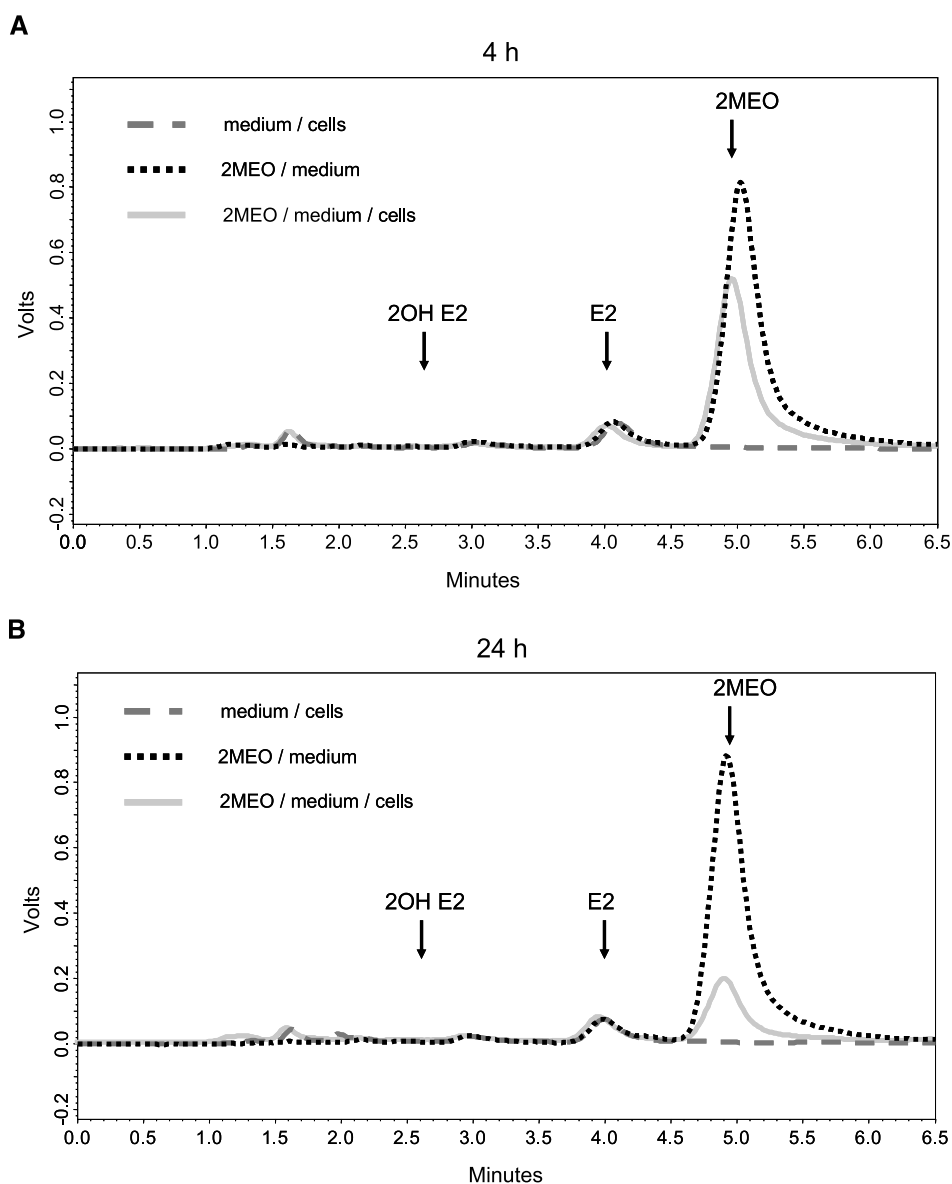
(10, 23, 42). More importantly, we have been repeatedly unable to confirm an antitumor growth effect of 2MEO.

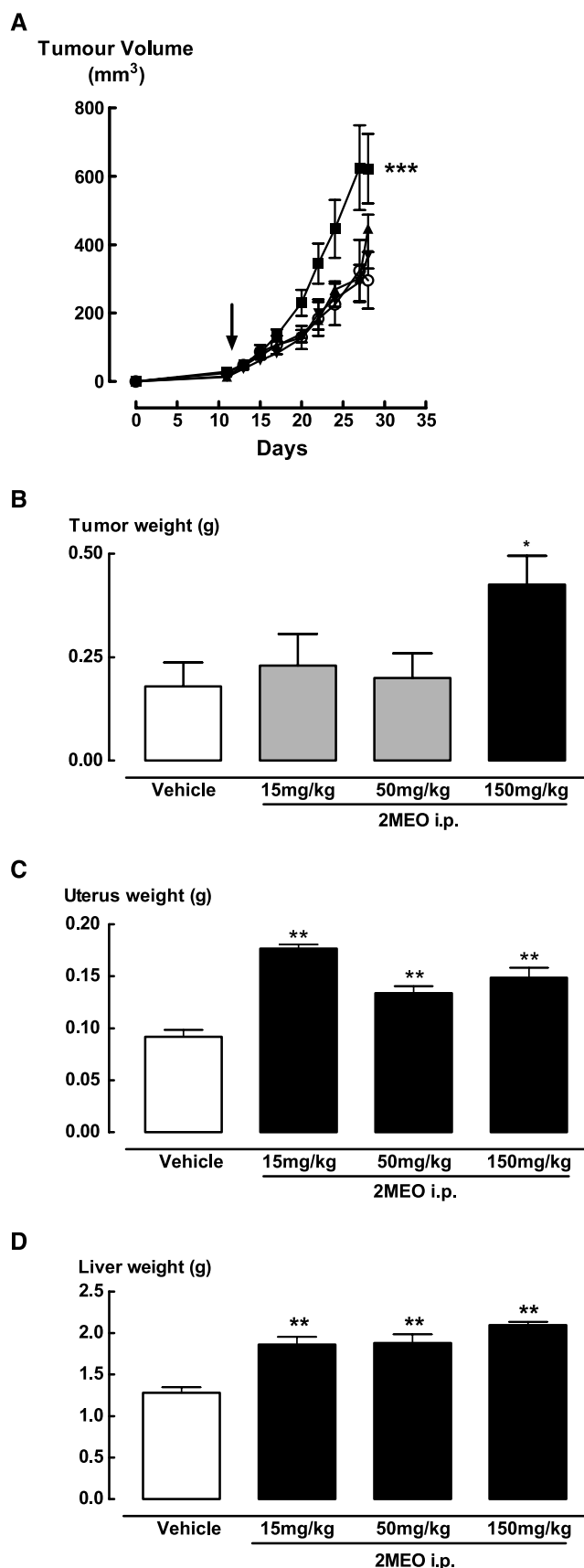
The notion that 2MEO lacks any significant activity at ER seems to have developed from an unwarranted extrapolation of comments made on radioligand binding data that showed that 2MEO did not bind to ER within the concentration range that had been reported physiologically (37) and that very low doses of 2MEO had no uterotrophic action (36). In addition, ER-binding studies have often been reported in terms of relative binding affinity in percentage terms (e.g., see refs. 19, 43–46). This method of expression can lead to misinterpretation, as it may be considered to represent the proportion of estrogen-like action that the agent may have, irrespective of its concentration. However, relative binding affinity denotes either the displacement activity of an analogue relative to that of E<sub>2</sub> at a fixed concentration or the relative affinities of the analogue and E<sub>2</sub> measured over a range of concentrations. In assessing the

likelihood of estrogenic action, two further considerations assume equal importance to the relative binding affinity: the physiologic or pharmacologic concentration range of the ligand and whether the ligand has agonist, antagonist, or mixed actions at the estrogen receptor. 2MEO has affinity for ER on the standard rat uterine cytosol preparation (18) and in intact MCF7 cells (35). The concentration of 2MEO occupying 50% of receptors (~100 nmol/L) is in the same order of magnitude as that for significant actions on either endothelial or tumor cells (100–1000 nmol/L; see, e.g., refs. 9, 19, 24, 35).

Having established that 2MEO or its metabolites would bind to a significant extent to ER when used at concentrations that are cytotoxic and antiangiogenic, we sought to examine whether 2MEO was an agonist or antagonist at ER. Antagonist actions, although advantageous in ER-positive breast tumors, would engender a range of adverse effects. Agonist actions would not only cause endocrine disruption, but could also facilitate growth

**Fig. 3** MCF7 cells were serum-deprived for 24 hours before addition of 3  $\mu\text{mol/L}$  2MEO and then incubated for either a further 4 (A) or 24 hours (B) at which time both the supernatant and the cell layer were extracted into acetonitrile. Medium/cells, extract of the normal culture medium (DMEM + 5% FCS) and confluent monolayer of MCF7 cells. 2MEO/medium, extract of the normal culture medium also containing 3  $\mu\text{mol/L}$  2MEO. 2MEO medium/cells, 2MEO-containing culture medium incubated with MCF7 cells. All incubations were carried out for either 4 or 24 hours. 2OH E<sub>2</sub>, 2-hydroxyestradiol.





of ER-positive tumors. Whereas enhanced tumor growth may be masked by ER-independent cytotoxic actions, such an interaction would at least have the potential to limit the efficacy and potency of the 2MEO in regulating growth of ER-dependent tumors. We used the MCF7 cell line to examine the ER agonist/antagonist actions of 2MEO. Analyses of early (4 hours) gene expression changes in response to 2MEO in comparison with E<sub>2</sub> indicated a high level of correlation in gene expression changes induced by these agents. Quantitative RT-PCR confirmed changes in microarray-detected changes in expression of a selection of genes including *CCND1*, *XBP-1*, *CD38*, and *PTGES*.

Complete concordance in action of E<sub>2</sub> and 2MEO was not expected, inasmuch as 2MEO is known to influence tubulin function independently of ER (25). Gene expression changes could be expected to result from this ER-independent action that would not be matched by E<sub>2</sub>-induced effects and could interfere with ER-dependent actions. For example, 2MEO decreases HIF-1 levels via disruption of microtubules resulting in down-regulation of *VEGF* gene expression (47). Notwithstanding such potentially confounding influence of tubulin actions, all but 10 of the 2MEO-induced changes in gene expression that were matched by E<sub>2</sub> changes (279 in total) were prevented by ICI 182,780. The down-regulation of *CD38* induced by either E<sub>2</sub> or 2MEO was insensitive to pretreatment with ICI 182,780, and the receptor mechanism for this effect warrants further investigation as *CD38* is a bifunctional enzyme (cyclic ADP ribosyl transferase/hydrolase) involved in the regulation of the release of calcium from intracellular stores. Moreover, decreased *CD38* expression has been implicated in apoptosis (48), raising the possibility that its decline in expression could contribute to the proapoptotic and antiproliferative actions of 2MEO in MCF7 cells. Our observation of an overlap in the genes responsive to E<sub>2</sub> and 2MEO is consistent with recent reports of E<sub>2</sub> and 2MEO gene expression changes in myeloma cells (49). Moreover the magnitude of the ER-dependent changes in gene expression are similar to those reported in other cell types (50).

Activation of ER-dependent changes in gene expression may occur through either transactivation- or transrepression-dependent mechanisms, the former requiring the presence of an ERE in the promoter sequence of the regulated gene and the latter resulting from ER/transcription factor interactions involving transcription factors such as nuclear factor  $\kappa$ B and C/EBP $\beta$  (51). Cyclin D1 regulation by E<sub>2</sub> has been ascribed to an ER interaction with cAMP-responsive element binding protein and activator protein 1 (52). Interestingly, much higher concentrations of 2MEO were required to increase cyclin D1 levels than to activate the ERE transfected into MCF7 cells (data not shown).

**Fig. 4** Dose-response relationships for the effects of 2MEO (15–150 mg/kg) given by i.p. injection on growth of MDA-MB-435 in *nu/nu* female BALB/c mice. 2MEO was given daily from when tumors were palpable (arrow) until the end of the experiment. Mice were treated with vehicle (○) or 2MEO (15 mg/kg, ▼; 50 mg/kg, ▲; 150 mg/kg, ■) given i.p. **A**, progressive tumor volume. **B**, postmortem tumor weights. **C**, uterus wet weight. **D**, liver wet weight. Points and columns, mean of five to eight mice; bars, SE. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; and \*\*\*,  $P < 0.001$  compared with vehicle-treated mice.

**Table 2** Effect of 2MEO (50 mg/kg/d i.p., 16 days) on liver and uterine weights in non-tumor-bearing BALB/c mice ( $n = 10$ )

Treatment	Uterus		Liver	
	Vehicle	2MEO	Vehicle	2MEO
Vehicle	0.098 ± 0.010	0.132 ± 0.009*	1.01 ± 0.03	1.37 ± 0.03*
ICI 182,780 (1 mg/kg/d i.p.)	0.052 ± 0.011	0.074 ± 0.006	0.99 ± 0.04	1.23 ± 0.04†

NOTE. Values are mean ± SE. Two-way ANOVA indicated that 2MEO increased both uterine and liver weight. Bonferroni post hoc testing was used to establish significance of individual contrasts.

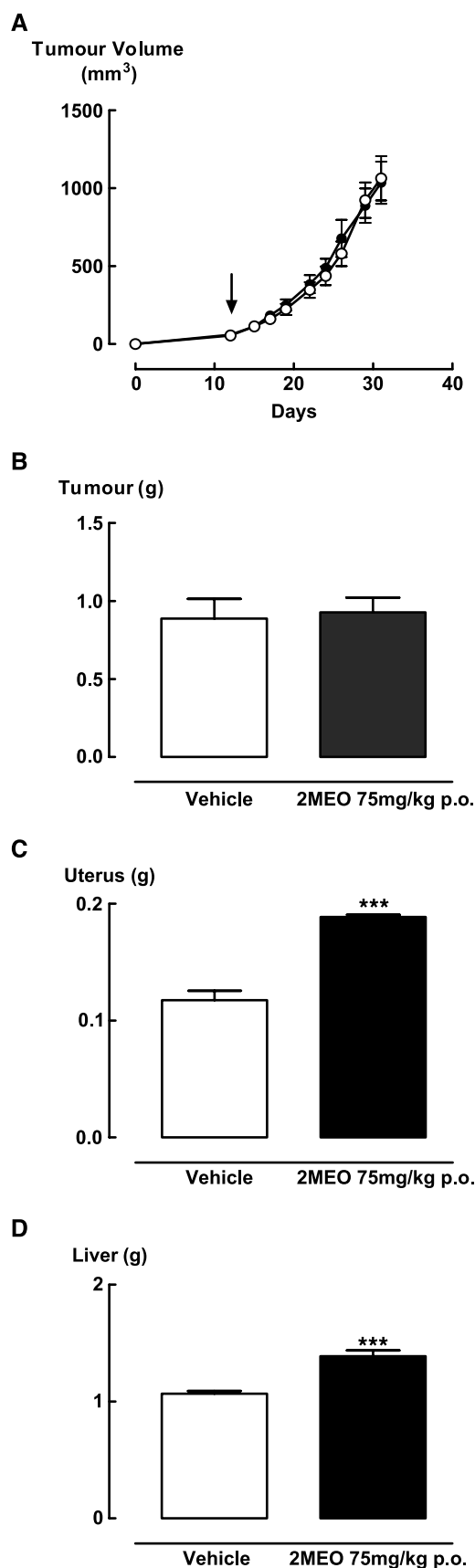
\* $P < 0.05$ , vehicle/vehicle versus vehicle/2MEO.

† $P < 0.05$ , ICI 182,780/vehicle versus ICI 182,780/2MEO.

A search of the 10,000-kb region extending up to the end of exon 1 for each of the  $E_2$ /2MEO-regulated genes revealed that none of the regulated genes has the perfect consensus ERE (5'-GGTCAnnnTGACC-3'). Many of the  $E_2$ -regulated genes have sequences that seem to be EREs, but vary in one or two nucleotides on one of the two arms of the palindromic ERE sequence (an imperfect ERE). Only EREs with less than three variations from the consensus sequence have been shown to be able to bind the ER homodimer (53, 54). *XBP-1* and *PTGES*, genes found to be responsive to both  $E_2$  and MEO, were found to have ERE sites that had one change from the consensus ERE. Other genes shown to be responsive to both MEO and  $E_2$  that contain at least one "imperfect" ERE include *FosB*, *AREG*, *FLTCL13*, *B3GALT3*, *CD38*, *GUCY1A3*, *HSD17B2*, and *FKBP4*. Finally, ERE half sites that contain one complete inverted repeat may also bind ER as part of a heteromeric complex and are often associated with other non-ERE elements such as activator protein 1 and Sp1. These sites have been identified in  $E_2$ -responsive genes such as the progesterone receptor and the proto-oncogenes *c-jun* and *c-fos* (55, 56).

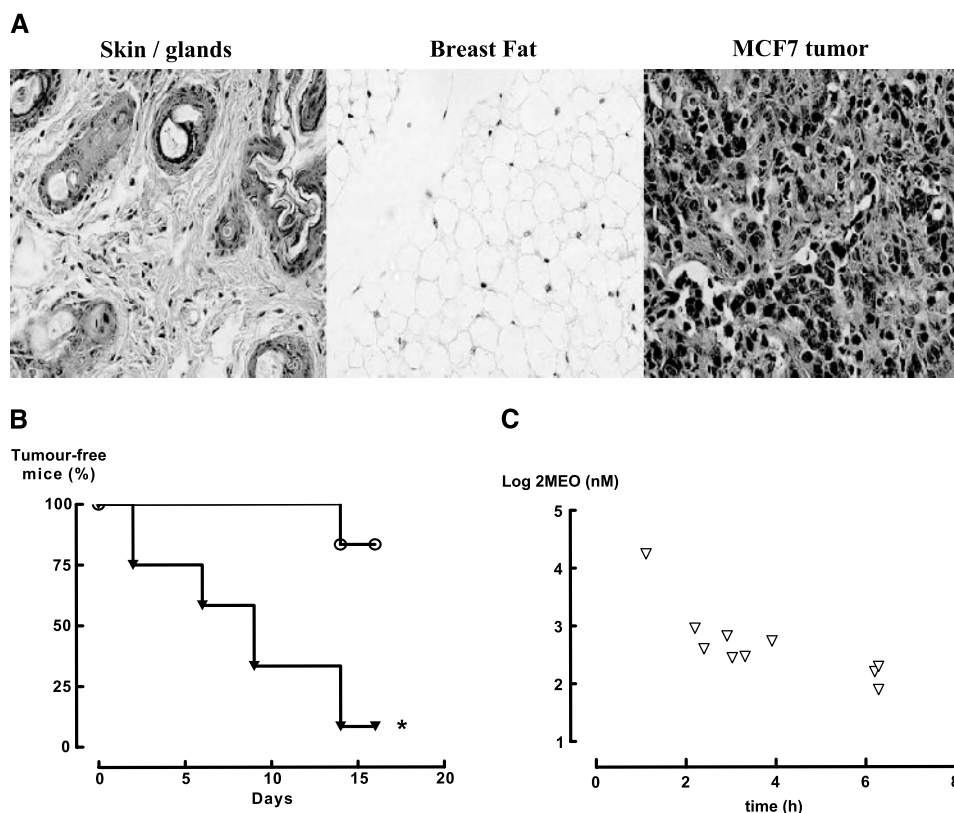
Collectively, our observations suggest that 2MEO engages ER to change gene transcription. Further evidence of ERE-type action was obtained from MCF7 cells transiently transfected with ERE upstream of a secreted alkaline phosphatase. 2MEO and  $E_2$  increases in activity of the ERE-dependent reporter gene were attenuated by pretreatment with ER antagonist, ICI 182,780. ICI 182,780, a complete ER antagonist, had a small but significant stimulatory effect on ERE activity that remains unexplained.

LaVallee et al. contend that 2MEO is not an agonist at ER, but their data suggest that 2MEO is converted to an ER agonist by MCF7 cells (35). When MCF7 cells were incubated with 2MEO (3  $\mu\text{mol/L}$ ) for up to 24 hours, there was no detectable formation of either 2-hydroxyestradiol or  $E_2$ , despite evidence of metabolism of 2MEO. Although shorter periods of incubation (4 hours), matching those of gene expression studies, showed that 2MEO levels fell by only 36%, we cannot exclude the



**Fig. 5** Effect of 2MEO (75 mg/kg, p.o., ●) compared with vehicle (0.5% carboxymethylcellulose, ○) given daily from day 14 to female SCID mice inoculated with  $10^6$  MDA-MB-435 cells into the flank at day 0. **A**, tumor growth was estimated by tumor volume. **B**, tumor growth results were confirmed by postmortem tumor weights. Uterine weight (**C**) and liver weight (**D**) determined at 31. Points and columns, mean of 11 to 12 mice; bars, SE. \*\*\*,  $P < 0.001$  compared with vehicle-treated mice.





**Fig. 6** Frequency of palpable tumors in vehicle- (10% DMSO/90% peanut oil, 100  $\mu$ L i.p.) and 2MEO- (50 mg/kg/d i.p.) treated female *nu/nu* BALB/c mice inoculated with MCF7 cells injected in Matrigel into the mammary fat pad. **A**, H&E-stained sections morphologically confirming the presence of MCF7 tumor (magnification  $\times 600$ ). **B**, frequency of palpable tumors was significantly different ( $P < 0.05$ ) between vehicle- ( $\circ$ ,  $n = 6$ ) and 2MEO- ( $\blacktriangledown$ ,  $n = 12$ ) treated mice. **C**, log of serum levels of 2MEO (nmol/L) in mice treated with 50 mg/kg/d for 16 days with serum being obtained at the indicated time (h) after the last dose. Each symbol represents the value from a single mouse.

possibility that a metabolite other than 2-hydroxyestradiol or  $E_2$  accounted for the ER stimulation. The proposed conversion of 2MEO to an ER-binding ligand by intact MCF7 cells at 37°C is unlikely to offer an explanation of binding of 2MEO to rat uterine cytosol at 4°C. Thus, the most plausible explanation of the available data is that 2MEO itself is an ER agonist at pharmacologic concentrations. Notwithstanding some uncertainty regarding the precise mechanism, there is unequivocal evidence from both the current study and that by LaVallee et al. to indicate that exposure of MCF7 cells to 2MEO results in ER-dependent responses.

Binding and activation of ER by 2MEO (or its metabolites) occur at concentrations required for antitumor actions *in vivo*. Thus, doses of 2MEO that decrease the growth and neovascularization of solid tumors were predicted to have estrogenic actions. The 2MEO-induced increases in uterine and liver weight confirmed our expectation of estrogen-related adverse effects (57–59). Furthermore, these estrogenic-like actions of 2MEO, also shown in non-tumor-bearing BALB/c mice, were attenuated by cotreatment with ICI 182,780. The latter observations confirmed that the estrogenicity of 2MEO was not dependent on the presence of the tumor or associated with the immune defect in the *nu/nu* BALB/c strain. Studies examining the estrogenic actions of compounds considered weak estrogens are often carried out in ovariectomized mice to preclude partial stimulation of estrogen-responsive tissues by endogenous estrogen that could mask the activity of weak estrogens. Our observations made in nonovariectomized mice reinforce the conclusion that pharmacologic doses of 2MEO are estrogenic in uterus and liver. Recent

studies in rats provide evidence that 2MEO has estrogen-like effects on bone cells and uterotrophic activity that were attenuated by ICI 182,780 treatment (60, 61).

Three separate experiments of adequate statistical power showed no detectable reduction of tumor growth by 2MEO. Furthermore, our observations indicate that 2MEO has estrogen-related toxicity at subtherapeutic doses (15–150 mg/kg/d). The protocols followed in our studies differ in several ways from those of previous reports in which antitumor effects of 2MEO had been observed. First, subjective measurements of tumor dimensions (tumor volume estimates) were carried out by an observer blinded to drug treatments, and on each occasion these measurements were confirmed by a second observer also blinded to drug treatment. Second, the tumors were inoculated into the mammary fat pad rather than *s.c.*, as this site of inoculation is of greatest relevance to breast cancer *in situ*. Third, 2MEO was given in a vehicle of DMSO/peanut oil rather than carboxymethylcellulose. Finally, tumor cells were injected in Matrigel to provide a small solid mass of cells and extracellular matrix, because this was considered to better simulate the form of tumor in its initial palpable and angiogenesis-dependent phase of development than injection of cells in a solution that would disperse in a more diffuse manner. Irrespective of the reasons for the failure to detect any impact of 2MEO on tumor growth, our observations stand in contrast to those of several (9, 10), but not all (15), previous investigations on the growth of the human breast tumor ER-negative MDA-MB-435 cell line. We can exclude the possibility of the development of 2MEO-resistant clone of the MDA-MB-435

cell line when maintained in our laboratory, inasmuch as we confirmed that the potency of 2MEO in decreasing the number of viable cells in culture (data not shown) was similar to that reported in previous studies (15, 43).

The failure to observe any antitumor actions at 2MEO doses having estrogenic actions prompted an attempt to replicate the conditions used by Klauber et al. (10). This experiment provided further confirmation of the estrogenic actions of 2MEO, but no evidence of an effect on tumor growth. If genetic drift in either the SCID mice or the MDA-MB-435 line explain the discrepancy between our study and that of Klauber et al., it would seem that the antitumor activity of 2MEO is highly dependent on the background of the tumor and the host, and therefore extrapolation of the reported activity in mice to human breast cancer is rendered even less reliable than it would be if antitumor actions were universal in murine models. In the context of phase I clinical evaluation of 2MEO in breast cancer, it is of some concern that 2MEO seemed to support the growth of the ER-dependent MCF7 tumor.

In summary, our findings lead to the conclusion that 2MEO is unsuitable as an antitumor agent because it lacks efficacy and has ER-dependent and ER-independent adverse effects. The unequivocal evidence of ER binding and agonist actions of 2MEO and its lack of antitumor efficacy raise concerns regarding its ongoing clinical evaluation as a novel treatment for breast and other forms of cancer. However, the well-confirmed antiangiogenic and tumor cell cytotoxic actions of 2MEO suggest that this agent should be considered as a prototype from which to develop lead agents with improved bioavailability and potency that are devoid of affinity for ER.

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