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Differential Effects of Estrone and Estrone-3-*O*-Sulfamate Derivatives on Mitotic Arrest, Apoptosis, and Microtubule Assembly in Human Breast Cancer Cells[1]

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ABSTRACT

There is considerable interest in the potential use of estrogen derivatives for the treatment and prevention of breast cancer. We demonstrated previously that the sulfamoylated estrone derivative 2-methoxyestrone-3-*O*-sulfamate (2-MeOEMATE) induced G_2-M cell cycle arrest and modest levels of apoptosis in breast cancer cells *in vitro*, whereas the parent estrone derivative, 2-methoxyestrone, did not. 2-MeOEMATE also induced breast tumor regression *in vivo* in intact rats. To further explore the significance of sulfamoylation on the anticancer activity of estrone derivatives and to elucidate their mechanism of action, we synthesized two additional agents, 2-ethylestrone and 2-ethylestrone-3-*O*-sulfamate (2-EtEMATE). 2-MeOEMATE and 2-EtEMATE inhibited the growth of a panel of estrogen receptor-negative and -positive breast cancer cell lines *in vitro*, induced mitotic arrest and apoptosis, and suppressed the long-term clonogenic potential of MCF7 and CAL51 breast cancer cells. In each assay, the sulfamoylated estrone derivatives were >10-fold more potent than their parent compounds. The sulfamoylated estrone derivatives were also significantly more potent inhibitors of cell growth than the previously studied endogenous estradiol metabolite 2-methoxyestradiol. 2-MeOEMATE and 2-EtEMATE functioned as antimicrotubule agents and inhibited the ability of paclitaxel to promote tubulin assembly *in vitro*. Like other antimicrotubule agents, the sulfamoylated estrone derivatives induced BCL-2 and BCL-X_L phosphorylation and increased p53 expression. 2-MeOEMATE and 2-EtEMATE are novel antimicrotubule agents that have potent anticancer activity in breast cancer cells *in vitro* and may be beneficial as anticancer agents *in vivo*.

INTRODUCTION

Estrogens undergo a series of hydroxylation, methylation, and conjugation reactions after their synthesis. Although these modifications were initially thought to be part of a metabolic process that enhanced their subsequent elimination from the body, it is now evident that at least some of the products of these reactions may possess unique activities mediated independently of the classical ER.[3] 2-MeOE2 is an estrogen-17β metabolite formed by sequential hydroxylation and methylation that does not bind ERs with high affinity (1) and has been the focus of considerable interest as an endogenous antimitotic factor. Zhu and Conney (2) have suggested that decreased endogenous levels of 2-MeOE2 create a predisposition to estrogen-induced cancer, and 2-MeOE2 is being studied as a potential therapeutic or preventative agent for breast cancer.

2-MeOE2 inhibits the growth of many cells, including human breast cancer lines, *in vitro* (3), and oral administration of 2-MeOE2 inhibits the growth of transplanted Meth-A sarcoma and B16 melanoma in C3H mice (4) and human MDA-MB-453 breast carcinoma cells in immunodeficient mice (5). 2-MeOE2 induces a reversible mitotic arrest (6) and apoptosis in retinoic acid-differentiated neuroblastoma SH-SY5Y cells and in human lung and pancreatic cancer cells (7–9). In addition, 2-MeOE2 inhibits endothelial cell proliferation and survival (10, 11) as well as migration and angiogenesis *in vitro* and *in vivo* (4, 5), and this may contribute to its anticancer activity.

The mechanism by which 2-MeOE2 induces mitotic arrest and/or apoptosis remains to be determined. 2-MeOE2 interacts with the colchicine binding site on β-tubulin and seems to induce a metaphase arrest by functioning as an antimicrotubule agent (12). However, the precise effects of 2-MeOE2 on tubulin have not been resolved entirely. Whereas several studies have demonstrated that 2-MeOE2 inhibits tubulin assembly *in vitro*, Attalla *et al.* (13) showed that, similar to paclitaxel, 2-MeOE2 promotes tubulin polymerization in intact cells. Although treatment of cells with 2-MeOE2 at concentrations that are relevant for biological effects does not cause gross disturbances of microtubule structures in cells, the same is probably true of other antimicrotubule agents, such as colchicine or paclitaxel. The underlying effect of these drugs at biologically relevant concentrations may be on the kinetics of mitotic spindle microtubule dynamics, rather than in the alteration of microtubule polymerization *per se* (14).

The induction of cell death by 2-MeOE2 may be mediated via effects on known apoptosis regulators such as BCL-2 or p53 (8, 13). BCL-2 suppresses apoptosis, and phosphorylation of BCL-2 is induced in K562 leukemic cells treated with 2-MeOE2 (13). This is likely to be a general response to antimicrotubule agents because BCL-2 is also phosphorylated in cells treated with paclitaxel or colchicine (14). In paclitaxel-treated cells, BCL-2 is phosphorylated (on Ser-70, Ser-87, and Thr-69), which leads to its inactivation (15). Phosphorylated BCL-2 is thought to be unable to bind and inactivate the proapoptotic BAX protein. Relatively high concentrations of 2-MeOE2 have also been shown to induce high-level expression of the p53 tumor suppressor, which is a potent inducer of apoptosis. In human lung cancer cell lines, wild-type p53 was required for efficient cell killing (8).

Although the *in vivo* and *in vitro* anticancer results obtained using 2-MeOE2 are encouraging, the bioavailability of estrogens that can be administered p.o. is generally poor. They are also subject to extensive modification during their first pass through the liver. As part of a research program to develop a steroid sulfatase inhibitor for breast cancer therapy, we identified the sulfamoylated estrone EMATE as an active site-directed inhibitor (16, 17). Surprisingly, EMATE proved to be potently estrogenic (18). This was thought to result from its absorption by RBCs, which protect it from inactivation and act as a reservoir for slow release *in vivo* (19). We therefore synthesized the A-ring-modified derivative, 2-MeOEMATE, which proved to be

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[3] The abbreviations used are: ER, estrogen receptor; 2-MeOEMATE, 2-methoxyestrone-3-*O*-sulfamate; 2-MeOE1, 2-methoxyestrone; 2-EtEMATE, 2-ethylestrone-3-*O*-sulfamate; 2-EtE1, 2-ethylestrone; 2-EtE2, 2-ethylestradiol; 2-MeOE2, 2-methoxyestradiol; EMATE, estrone-3-*O*-sulfamate; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; PI, propidium iodide.

nonestrogenic (20). However, we noticed that, when added to cells, 2-MeOEMATE was a potent inhibitor of cell growth of ER-positive MCF7 and ER-negative MDA-MB-231 breast cancer cells, and it induced a G_2-M arrest and modest levels of apoptosis in MCF7 cells (21). In a small *in vivo* study, oral administration of 2-MeOEMATE caused the rapid regression of nitrosomethylurea-induced mammary tumors in intact rats (21). Interestingly, the nonsulfamoylated estrone 2-MeOE1 was considerably less effective in these assays.

These data suggested that, like 2-MeOE2, certain conjugated-estrone derivatives such as 2-MeOEMATE have potent growth-inhibitory activity. Importantly, sulfamoylation at the 3-position of the A-ring of estrones may be a useful way of increasing the growth-inhibitory properties and bioavailability of these molecules. The aims of this study were to identify additional conjugated estrone derivatives with potent anticancer activity, further explore the significance of the sulfamate modification, and understand the mechanism of action of the estrone derivatives.

MATERIALS AND METHODS

Drug Synthesis. 2-MeOEMATE was synthesized as described (19, 20). 2-MeOE2 and 2-MeOE1 were purchased from Sigma Chemical Co. Briefly, 2-EtE1 and 2-EtEMATE were prepared from estrone by Friedel-Crafts acetylation of estrone-3-*O*-methyl ether and catalytic hydrogenation, followed by demethylation, which produced 2-EtE1. This was reacted with sulfamoyl chloride to obtain the corresponding 2-EtEMATE. 2-EtE2 has been synthesized previously by a different route (22). All compounds exhibited spectroscopic and analytical data in accordance with their structure. Full details of the synthesis process will be reported elsewhere. Compounds were prepared as 10 mM stocks in tetrahydrofuran.

Cell Culture. MCF7, CAL51, CAMA1, and ZR-75-1 human breast cancer-derived cell lines were obtained from American Type Culture Collection (MCF7, CAMA1, and ZR-75-1) or from the Dutrillaux laboratory (CAL51; Ref. 23), maintained in DMEM containing phenol red, and supplemented with 10% (v/v) FCS and antibiotics. The approximate passage numbers are as follows: MCF7, 320; CAL51, 80; ZR-75-1, 40; and CAMA1, 60. Paclitaxel was obtained from Bristol-Meyers Squibb Co. The effects of the drugs on cell growth were determined by cell counting using a Coulter counter (Fig. 6) or by a microtiter plate assay (Cell Titer 96 cell proliferation assay; Promega; Fig. 2). In the experiments using the microtiter plate assay, the conversion of substrate by untreated cells at the end of the culture period was set at 100%. In colony formation experiments, colonies were fixed using methanol and stained using Wright-Giemsa.

DNA content and apoptosis were determined by flow cytometric analysis of PI-stained cells and by TUNEL, respectively, as described (21). The proportion of cells in the G_2-M phase of the cell cycle was calculated as a proportion of cells with 2 N to 4 N DNA content. The proportion of cells with sub-G_1 DNA content was calculated as the percentage of total cells.

To determine the proportion of cells in mitosis, drug-treated cells were collected by trypsinization, and cytospins were prepared. Cells were fixed in ice-cold methanol for 5 min and air dried, and DNA was stained using PI [0.1 mg/ml in PBS containing 10% (v/v) newborn calf serum and 0.05% (w/v) sodium azide]. Cells were analyzed by confocal microscopy using a Zeiss Axiovert 100M microscope equipped with the LSM 510 confocal system (Zeiss, Jena, Germany).

Western Immunoblotting and Immunofluorescence. Western blotting was performed as described previously (24) using the following antibodies: anti-BCL-X_L (R&D Systems, Inc.); anti-BCL-2 (C124; DAKO); anti-p53 (DO-1); and anti-β-tubulin (Sigma Chemical Co.). Tubulin fractionation was performed as described (25). The loading for the immunoblot shown in Fig. 7 was normalized for equal cell numbers at the beginning of the experiment. The loading for the immunoblot shown in Fig. 10 was normalized for total protein content (24).

For immunofluorescence analysis of microtubule structures, MCF7 cells were allowed to adhere to BioCoat collagen I-coated glass slides (Becton Dickinson) overnight and subsequently drug treated for 48 h. Cells were washed in PBS and fixed in 3.5% (w/v) paraformaldehyde:2% (w/v) sucrose in PBS in a 1:1 solution of DMEM at room temperature for 10 min. Cells were permeabilized on ice for 5 min with 20 mM HEPES, 300 mM sucrose, 50 mM NaCl, 3 mM $MgCl_2$, 0.5% (v/v) Triton X-100, and 0.05% (w/v) sodium azide (pH 7.0), followed by primary anti-β-tubulin staining (1:200 dilution), washing, and secondary antimouse FITC staining (1:40 dilution; DAKO). Confocal images were collected using the Zeiss LSM 510 confocal microscope.

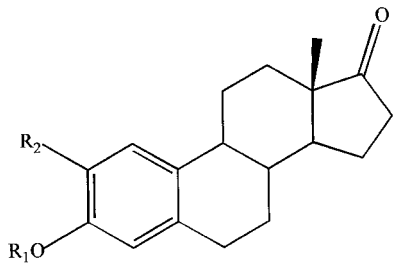
Tubulin Turbidimetry. *In vitro* tubulin assembly was measured by turbiditry at 340 nm. Tubulin (Cytoskeleton, Inc., Denver, CO) at a final concentration of 1 mg/ml was incubated at 32°C in G-PEM buffer [80 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) sequisodium salt, 1 mM $MgCl_2$, 1 mM EGTA, and 1 mM GTP (pH 6.8)] in the presence or absence of paclitaxel or estrones derivatives.

RESULTS

Short-Term Growth Assays. We demonstrated previously that the sulfamoylated estrone, 2-MeOEMATE, but not the parent compound, 2-MeOE1, potently inhibited the *in vitro* growth of MCF7 breast cancer cells (21). In an attempt to further explore the significance of sulfamoylation and identify additional estrone derivatives with growth-inhibitory properties, we synthesized 2-EtE1, in which the methoxy group in 2-MeOE1 is replaced by an ethyl group and its sulfamoylated derivative, 2-EtEMATE (Fig. 1).

To compare the effects of these molecules on breast cancer cell growth, MCF7 cells were treated with different concentrations of the compounds for 4 days, and cell growth was measured using a microtiter plate assay (Fig. 2*a*). 2-MeOE1 and 2-EtE1 had no effect on the growth of MCF7 cells, even at the highest dose tested (5 μM). By contrast, 2-MeOEMATE and 2-EtEMATE significantly inhibited MCF7 cell growth (maximal inhibition, approximately 50–70%). Therefore, similar to the sulfamoylation effects of 2-MeOE1 (21), this modification also potently enhances the growth-inhibitory activity of 2-EtE1. Because the estradiol derivative 2-MeOE2 has also been shown to possess anticancer activity in MCF7 cells (3), we compared the ability of this compound to inhibit cell growth with 2-MeOE1 and 2-MeOEMATE (Fig. 2*e*). Although 2-MeOE2 inhibited cell growth to the same extent as 2-MeOEMATE at relatively high concentrations (5 μM), it was at least 10-fold less effective than 2-MeOEMATE in this assay.

To determine whether the potent growth-inhibiting activities of the sulfamoylated compounds were specific to MCF7 cells, we tested the effects of the drugs on additional breast cancer cell lines: ZR-75-1, CAL51, and CAMA1 (Fig. 2, *b*–*d*). In each cell line, growth was significantly inhibited by 2-MeOEMATE and 2-EtEMATE but not by



R_1	R_2	
H	OMe	2-MeOE1
SO_2NH_2	OMe	2-MeOEMATE
H	Et	2-EtE1
SO_2NH_2	Et	2-EtEMATE

Fig. 1. Structures of 2-MeOEMATE, 2-MeOE1, 2-EtEMATE, and 2-EtE1.

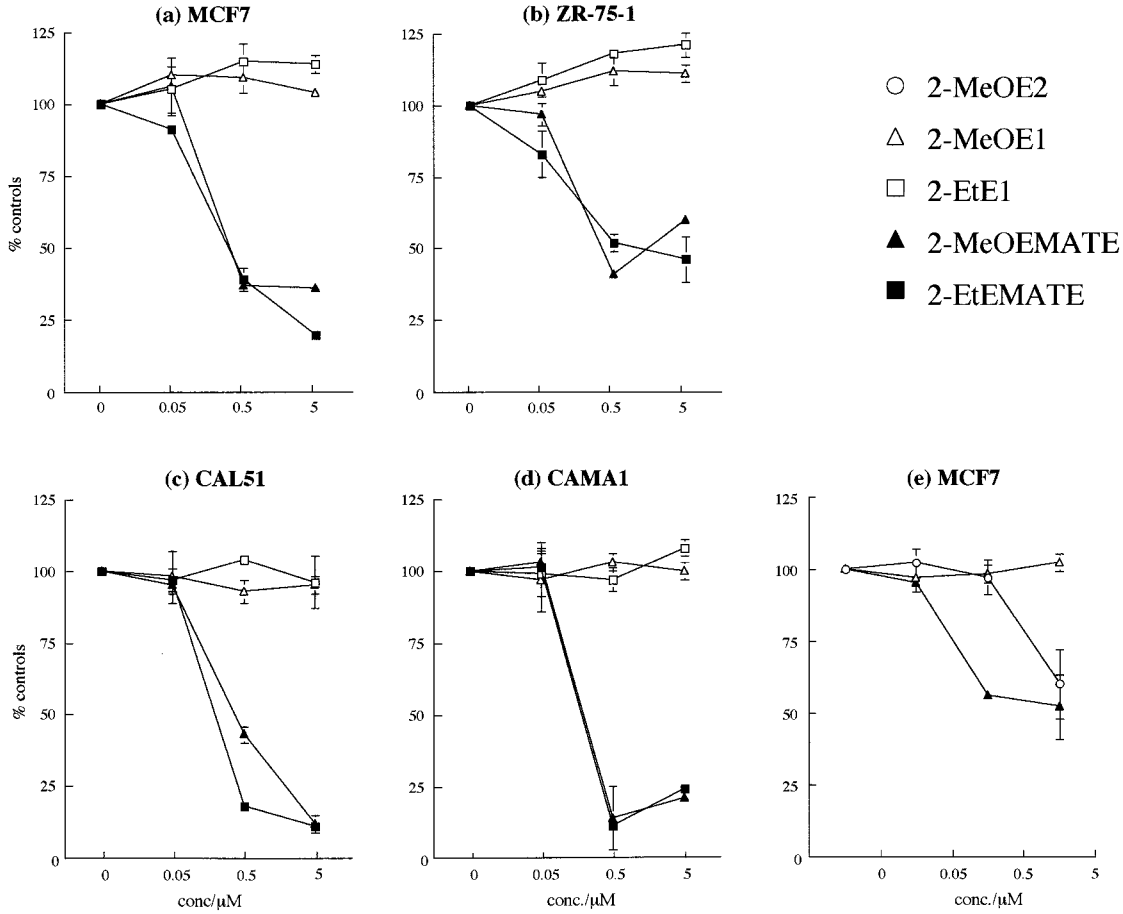


Fig. 2. Effect of estrone and estradiol derivatives on human breast cancer cell lines. MCF7, ZR-75-1, CAL51, and CAMA1 cells were treated with the indicated compounds, and the effect on cell growth was determined after 4 days using a microtiter plate assay. Values are means of triplicate determinations; *bars*, SD (some errors are too small to show). Values obtained in untreated wells at the end of the experiment were used to derive percentages. Results presented are representative of at least three experiments.

the parent compounds. Interestingly, there were differences in the relative sensitivity of the cell lines to these drugs. MCF7 and ZR-75-1 cells were relatively resistant to the sulfamoylated estrones (50–70% maximal inhibition) compared with CAL51 and CAMA1 cells (~90% maximal inhibition). CAL51 cells do not express detectable levels of ERs (23), confirming our previous observation (using ER-negative MDA-MB-231 cells) that the potent growth-inhibitory activity of sulfamoylated estrones does not require ERs (21).

Because MCF7 and ZR-75-1 cells are ER positive and were cultured in the presence of estrogens (complete serum- and phenol red-containing medium), it was possible that signals mediated by the ER contributed to their relative resistance to the growth-inhibitory effects of 2-MeOEMATE and 2-EtEMATE. Therefore, we tested the effect of ER directly on growth inhibition by sulfamoylated estrones. MCF7 cells were treated with ICI 182,780 (a pure, nonsteroidal antiestrogen) at 100 nM for 2 days to inhibit ER function or left untreated as a control and then treated for 4 days with 2-MeOEMATE or 2-EtEMATE in the continued presence of ICI 182,780. However, inhibition of ER function did not sensitize MCF7 cells to growth inhibition by either compound (data not shown). The effectiveness of ICI 182,780 treatment was confirmed by immunoblotting for BCL-2, a transcriptional target of the ER. BCL-2 was down-regulated in ICI 182,780-treated cells (data not shown). Therefore, ER-dependent signaling does not contribute to the resistance to sulfamoylated estrone derivatives in MCF7 cells.

Cell Cycle and Apoptosis Analysis. We examined whether the differential sensitivity of breast cancer cell lines to the growth-inhibitory effects of sulfamoylated estrones was related to differences in the extent/phase of cell cycle arrest and/or cell death. These studies focused on 2-EtEMATE because we were able to readily synthesize relatively large amounts of this compound. We first examined the effects of 2-EtEMATE on the DNA content of MCF7, ZR-75-1, CAL51, and CAMA1 cells using flow cytometry of PI-stained cells (Table 1). Cells were treated with 2-EtEMATE at 500 nM, because this was the lowest dose that effected significant growth inhibition in each cell line (Fig. 2). MCF7 cells accumulated in the G_2-M phase of the cell cycle within 24 h, and cells were maximally arrested (~60% of cells) after 48 h. This arrest was maintained for the duration of the experiment, although there was a modest increase in cells with sub-G_1 DNA content, which was indicative of cell death at 72 and 96 h. Similar results were obtained in ZR-75-1 cells, although G_2-M arrest was delayed in these cells (60% of cells in G_2-M after 72 h). By contrast, CAL51 and CAMA1 cells, which were more sensitive than MCF7 or ZR-75-1 cells in the microtiter plate assay (Fig. 2), underwent a more rapid G_2-M arrest (~60% of cells were in G_2-M within 24 h). In addition, 2-EtEMATE induced significant cell death within 48 h, and approximately half of the cells were dead after 96 h (Table 1).

Flow cytometric analysis demonstrated that cells treated with the sulfamoylated estrone derivatives were arrested in G_2-M phases of the cell cycle. Cells treated with 2-EtEMATE or 2-MeOEMATE often had a characteristic “rounded-up” morphology (Ref. 21 and data not shown), suggesting that cells were, in fact, arrested in mitosis. To determine whether cells were in interphase or mitosis, we stained drug-treated MCF7 cells with PI to visualize chromosomes. Cells

Table 1 *Effect of 2-EtEMATE on DNA content of breast cancer cell lines*

Cell cycle parameters of MCF7, ZR-75-1, CAL51, and CAMA1 cells exposed to 2-EtEMATE (500 nM) for up to 96 h are shown. The proportion of cells in G_1-S or G_2-M are shown as a percentage of total cells with a 2 N to 4 N DNA content. The proportion of cells with a sub-G_1[a] DNA content is shown as a percentage of total cells. Untreated cells are at 96 h.

	MCF7			ZR-75-1			CAL51			CAMA1		
Cell line	$<G_1$	G_1-S	G_2-M	$<G_1$	G_1-S	G_2-M	$<G_1$	G_1-S	G_2-M	$<G_1$	G_1-S	G_2-M
Untreated	2	77	23	1	79	20	1	78	22	4	70	30
24 h	5	63	37	1	84	15	2	38	62	4	33	67
48 h	5	38	62	6	60	40	10	18	82	16	25	75
72 h	12	34	66	12	37	63	21	32	68	36	33	67
96 h	8	29	71	9	20	80	44	23	77	51	33	67

[a] $<G_1$, sub-G_1.

were treated with 500 nM or 5 μM of the compounds for 24 h, because the flow cytometric analysis showed significant accumulation of cells in G_2-M at this time (Table 1). In preliminary experiments, we found that the rounded-up cells did not fix to the collagen-treated glass slides used for fluorescence staining experiments; therefore, we collected all of the cells by trypsinization and prepared cytospins before staining. The vast majority of control MCF7 cells had a uniformly stained nucleus characteristic of interphase cells with uncondensed chromosomes (Fig. 3 and Table 2). By contrast, a significant proportion of cells treated with 2-EtEMATE or 2-MeOEMATE showed condensed chromosomes characteristic of mitosis. Therefore, the sulfamoylated estrone derivatives induced a mitotic arrest. Consistent with the lack of effect in the flow cytometric assay (Table 1), the nonsulfamoylated estrone derivative, 2-EtE1, did not increase the number of mitotic cells. Because it was necessary to use cytospins in these experiments,

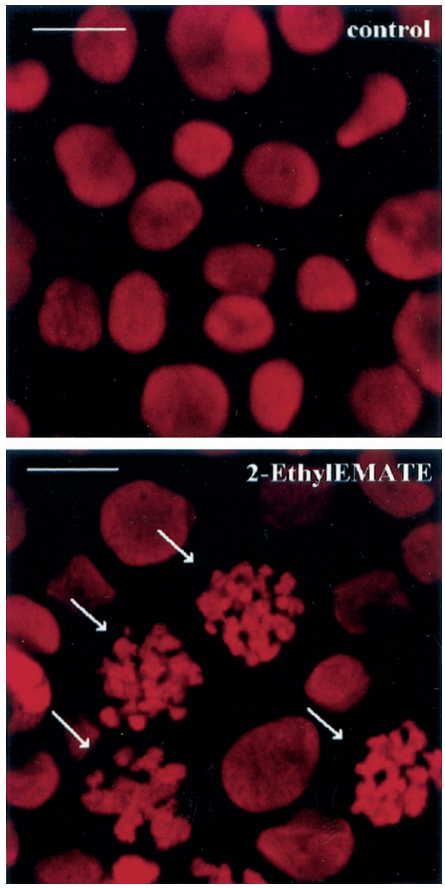


Fig. 3. Mitotic arrest induced by sulfamoylated estrone derivatives. MCF7 cells were treated with 2-EtMATE (5 μM) for 24 h or left untreated as a control. Cells were collected by trypsinization, cytospins were prepared, and DNA was stained using PI (for quantitation, see Table 2). The *bar* in the *upper left corner* of each image is equivalent to 20 μm. *Arrows*, cells with condensed chromosomes.

Table 2 *Effect of estrone derivatives on mitosis in MCF7 cells*

MCF7 cells were treated with the indicated compounds for 24 h. Cells were recovered by trypsinization, and cytospins were prepared. DNA was stained with PI, and cells in mitosis (*i.e.*, with condensed chromosomes) were determined as a percentage of total cells.

Compound	Mitotic cells
Control	4%
2-EtEMATE, 5 μM	49%
2-EtEMATE, 500 nM	29%
2-MeOEMATE, 5 μM	38%
2-MeOEMATE, 500 nM	26%
2-EtE1, 5 μM	6%
2-EtE1, 500 nM	4%

it was difficult to determine the architecture of the chromosomes in cells treated with sulfamoylated estrone derivatives. However, the chromosomes seemed to be fully condensed, suggesting that the cells had reached prometaphase/metaphase.

We used the TUNEL assay to confirm that the cell death detected by flow cytometry of PI-stained cells was attributable to apoptosis. Cells were treated with 500 nM 2-EtEMATE for 72 h because flow cytometric analysis demonstrated significant cell death at this time (Table 1). After exposure to 2-EtEMATE, there was a significant increase in the proportion of CAL51 and CAMA1 cells undergoing apoptosis (60 and 37% TUNEL-positive cells, respectively; Fig. 4). By contrast, there was only a modest increase in TUNEL positivity in MCF7 or ZR-75-1 cells (7 and 10%, respectively) treated with 2-EtEMATE at this concentration.

Taken together, these analyses demonstrate that the variations in the sensitivity of breast cancer cell lines to short-term growth inhibition by sulfamoylated estrones are reflected in differences in the effects on the cell cycle and apoptosis. Although 2-EtEMATE induced a G_2-M arrest in all cell lines, this was more rapid in the relatively sensitive CAL51 and CAMA1 cells than in the MCF7 and ZR-75-1 cells. Furthermore, 2-EtEMATE was a more potent inducer of apoptosis in CAL51 and CAMA1 cells than it was in MCF7 and ZR-75-1 cells.

Long-Term Growth Assays. To test whether growth-inhibitory effects of sulfamoylated estrones were also evident in long-term assays, we determined the effect of 2-EtE1, 2-EtEMATE, 2-MeOE1, and 2-MeOEMATE on the clonogenic potential of MCF7 cells. MCF7 cells were exposed to the different compounds (at 10 μM) for 4 days, and the number of viable cells remaining was determined. Equal cell numbers were plated at low density in the absence of any drug, and colonies were allowed to form for 13 days (Fig. 5). Transient treatment with 2-EtE1 or 2-MeOE1 had little effect on the long-term cloning efficiency (32 $\pm$ 2% and 42 $\pm$ 2%, respectively, *versus* control cells, 26 $\pm$ 5%). Consistent with their short-term growth-inhibitory effects, treatment with 2-EtEMATE or 2-MeOEMATE resulted in somewhat reduced recovery of viable cells after 4 days. However, in contrast to cells treated with the nonsulfamoylated compounds, the cloning efficiency of the remaining viable cells was very low (1.4 $\pm$ 0.6% and 2.1 $\pm$ 0.4%, respectively).

A similar experiment was performed on CAL51 cells treated with

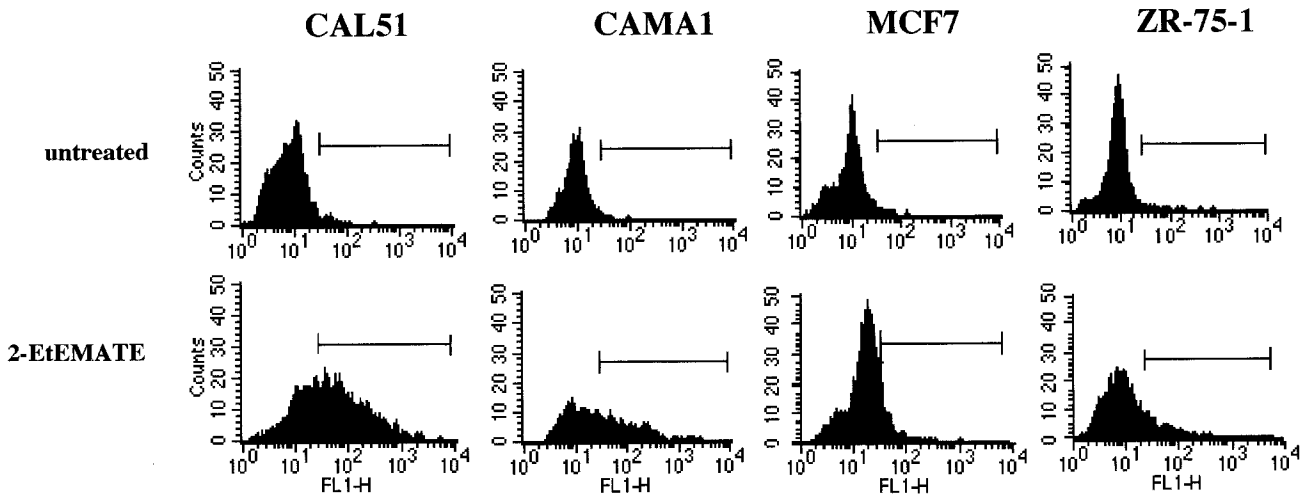


Fig. 4. Induction of apoptosis by sulfamoylated estrone derivatives. MCF7, ZR-75-1, CAL51, and CAMA1 cells were treated with 2-EtEMATE (500 nM) for 72 h or left untreated as a control. Apoptotic cells were identified using the TUNEL assay. The *marker* indicates cells considered as TUNEL-positive for each cell line. Results presented are representative of two experiments.

2-EtE1 or 2-EtEMATE at 500 nM for 2 days. A lower concentration of the drug and a shorter incubation time were used in this experiment because CAL51 cells were more sensitive than MCF7 cells (Fig. 2). As before, the cloning efficiency of CAL51 cells exposed to 2-EtE1 (22 ± 7%) was very similar to untreated cells or to cells exposed to solvent as a control (33 ± 8% and 30 ± 5%, respectively). By contrast, transient exposure to 2-EtEMATE significantly reduced the clonogenic potential of CAL51 cells (to 1.0 ± 1.1%). Therefore, short-term exposure to 2-MeOEMATE or 2-EtEMATE, but not to the nonsulfamoylated parent compounds, significantly reduces (by at least 95%) the clonogenic potential of MCF7 and CAL51 cells.

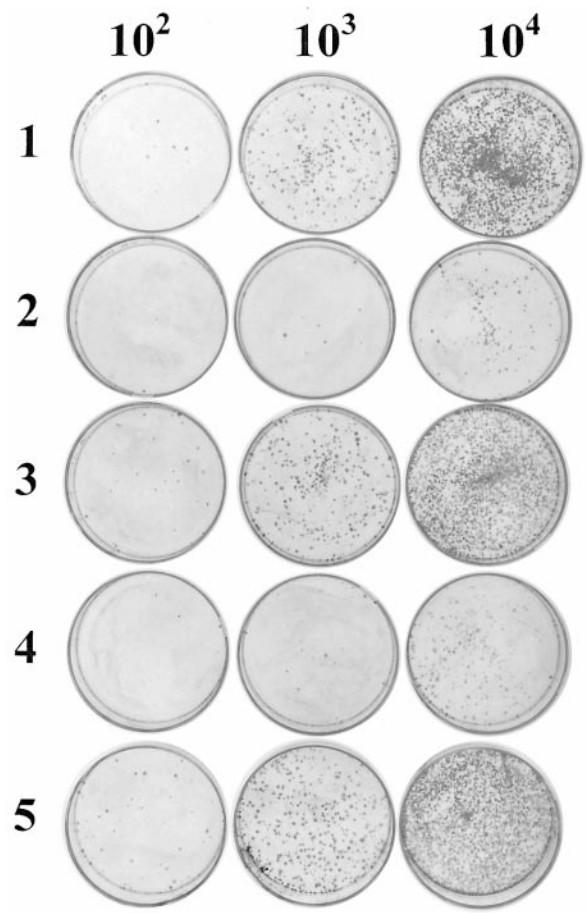


Fig. 5. Effect of transient exposure to estrones on MCF7 clonogenic potential. MCF7 cells were treated with 2-EtEMATE (*row 2*), 2-EtE1 (*row 3*), 2-MeOEMATE (*row 4*), or 2-MeOE1 (*row 5*), all at 10 μM, or left untreated as a control (*row 1*). After 96 h, cells were washed thoroughly, and viable cell numbers were determined. Cells ($\times 10^4$, $\times 10^3$, or $\times 10^2$) were plated on 6-cm plates, and colonies were allowed to grow for 13 days before staining with Wright-Giemsa. See text for quantitation.

To test whether these long-term growth-inhibitory effects were attributable to irreversible inhibition of cell proliferation or to delayed cell death, MCF7 cells were exposed to 2-EtEMATE, 2-MeOEMATE, or 2-EtE1 (at 10 μM) for 3 days. The cells were then washed to remove the drug and cultured in the absence of the drug for an additional 4 days. Cell numbers were determined after 3 days of drug exposure and after 4 days of drug-free incubation (Fig. 6). Although cell numbers were lower after exposure to 2-EtE1, the subsequent rate of cell growth in drug-free medium was similar to that of untreated MCF7 cells. By contrast, cell numbers continued to decline during the 4-day drug-free incubation period in cells exposed previously to 2-MeOEMATE or 2-EtEMATE. Therefore, 2-MeOEMATE and 2-EtEMATE inhibit the long-term growth of breast cancer cells by irreversibly committing the cells to cell death. Similar to the nonsulfamoylated estrones, the growth-inhibitory effects of 2-MeOE2 in MCF7 cells were reversible (Fig. 6).

Effect on Tubulin Polymerization. Because the sulfamoylated estrone derivatives induced mitotic arrest, we thought they might function as antimicrotubule agents. Known antimicrotubule agents, such as paclitaxel or colchicine, promote or inhibit tubulin polymerization, respectively. A portion of the tubulin in paclitaxel-treated cells is resistant to mild detergent extraction (26), and 2-MeOE2 has also been suggested to promote tubulin polymerization (13). We therefore tested whether the sulfamoylated estrone derivatives promoted tubulin polymerization in intact cells. We treated MCF7 cells with 2-EtEMATE or 2-EtE1 (at 500 nM or 5 μM) and determined the relative abundance of soluble and insoluble β-tubulin (Fig. 7). Cells were analyzed after 48 h because they underwent a profound mitotic arrest at this time (Table 1). However, there was no increase in the proportion of β-tubulin recovered in the insoluble fraction. By contrast, ~50% of the tubulin in cells treated with paclitaxel at 500 nM was recovered in the insoluble fraction. Because relatively low concentrations of paclitaxel (50 nM) induced G_2-M arrest in MCF7 cells (data not shown), it was important to examine the effects of paclitaxel

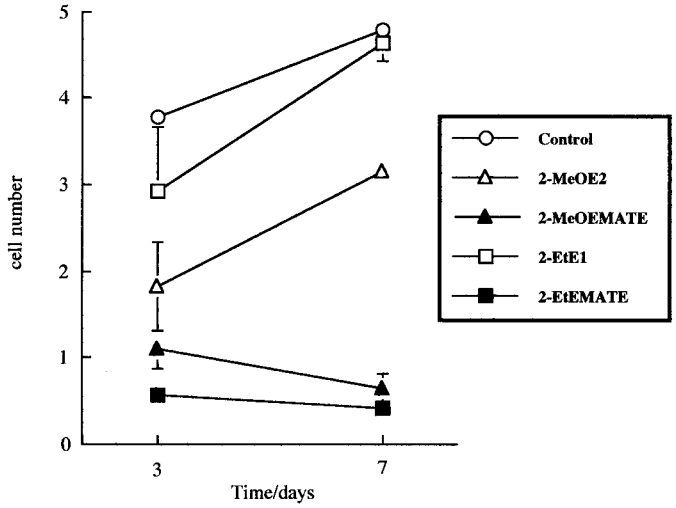


Fig. 6. Reversibility study. MCF7 cells were exposed to the indicated compounds (10 μM) for 3 days, and cell numbers were determined by Coulter counting. Cells were washed thoroughly to remove drugs, and cell numbers ($\times 10^6$) were then determined after a 4-day, drug-free incubation. Values are means of triplicate determinations; *bars*, SD (some errors are too small to show).

on tubulin polymerization at lower concentrations. However, treatment of MCF7 cells with concentrations of paclitaxel that were not sufficient to induce G_2-M arrest (5 nM) still resulted in the formation of insoluble tubulin. Therefore, in contrast to the microtubule-stabilizing agent paclitaxel, the sulfamoylated estrone derivatives do not promote tubulin polymerization in intact cells.

We further explored the effect of the sulfamoylated estrone derivatives on tubulin polymerization using purified tubulin and turbidimetry. As shown previously (26), paclitaxel promoted efficient *in vitro* microtubule assembly, and the rate of assembly was not effected by tetrahydrofuran, the solvent used for the estrone derivatives (Fig. 8, *a* and *b*). Consistent with the results in MCF7 cells, 2-EtEMATE alone did not promote tubulin assembly over short times (Fig. 8*c*) or for 1.5 h (data not shown). However, prior addition of 2-EtEMATE significantly decreased (by ~70%) the maximal rate of polymerization induced by the subsequent addition of paclitaxel. This was dose dependent; the rate of paclitaxel-induced polymerization in the presence of 1 μM 2-EtEMATE was similar to that of the solvent control (Fig. 8*c*). Similar to 2-EtEMATE, 2-MeOEMATE did not promote tubulin assembly but did significantly decrease the subsequent rate of paclitaxel-induced polymerization (Fig. 8*e*). The nonsulfamoylated compounds had no effect on tubulin assembly in the presence or absence of paclitaxel (Fig. 8, *d* and *f*). Therefore, the sulfamoylated estrone derivatives inhibit tubulin polymerization *in vitro*. Although relatively high concentrations of 2-MeOE2 (>20 μM) have been shown previously to inhibit glutamate-induced tubulin assembly *in vitro* (12), we were unable to demonstrate a significant effect of this compound at the concentrations tested in this assay (Fig. 8*g*).

We also determined the effects of the sulfamoylated estrones on microtubule structures in cells. MCF7 cells were cultured on collagen-coated glass slides in the presence or absence of estrones. The cells were fixed, and β-tubulin was detected by immunofluorescence (Fig. 9). Because rounded-up cells are not fixed onto the slides, we were able to specifically analyze the effects on the cells in interphase. Cells were analyzed after 16 h because previous studies of microtubule-disrupting agents have shown effects within this time frame (6). In control cells, most cells had a characteristic microtubule network that excluded the nucleus and extended throughout the cytoplasm. The microtubule network was not disrupted in MCF7 cells exposed to the sulfamoylated estrone derivatives, although a relatively high concentration of compound was used in these experiments (5 μM). Equivalent results were also obtained when cells were treated for longer times (data not shown). Therefore, although sulfamoylated estrone derivatives inhibit tubulin polymerization *in vitro*, their potent antimitotic activity is not associated with gross alterations in microtubule structures in interphase cells.

Effect on Apoptosis Regulators. Because the sulfamoylated estrone derivatives were potent inducers of apoptosis in some breast cancer cells, we tested their effects on the expression of BCL-2 family proteins and p53. Antimicrotubule agents such as paclitaxel, the *Vinca* alkaloids, and colchicine have been shown to induce phosphorylation of BCL-2 and BCL-X_L and to stabilize wild-type p53 (8, 14, 15, 27). MCF7, ZR-75-1, CAL51, and CAMA1 cells were treated with different concentrations of 2-EtEMATE, 2-EtE1, and paclitaxel as a positive control for 24 h. Although the expression of BCL-X_L was relatively consistent between cell lines in untreated cells, we detected significant variation in BCL-2 levels. BCL-2 expression was highest in MCF-7 and ZR-75-1 cells and lowest in CAL51 and CAMA1 cells. Phosphorylation of BCL-2 and BCL-X_L (as determined by the ap-

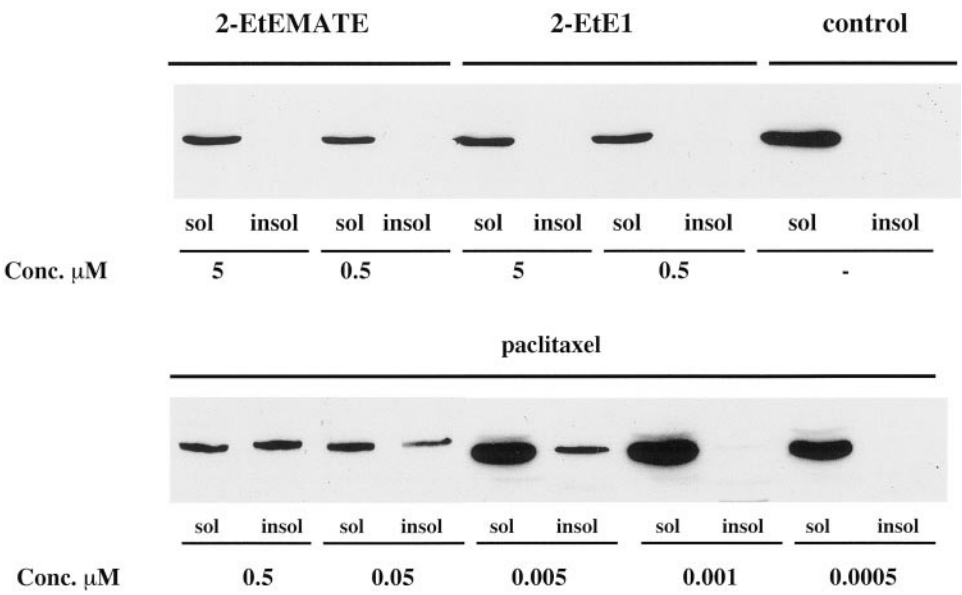


Fig. 7. Effect of 2-EtEMATE, 2-EtE1, and paclitaxel on tubulin polymerization in MCF7 cells. MCF7 cells were treated with 2-EtEMATE, 2-EtE1, or paclitaxel at the indicated concentrations for 48 h or left untreated as a control. Soluble (*sol*) and insoluble (*insol*) tubulin were separated by fractionation, and β-tubulin was detected by immunoblotting. Total β-tubulin is reduced in cells exposed to high concentrations of paclitaxel as a result of high levels of cell death/growth inhibition.

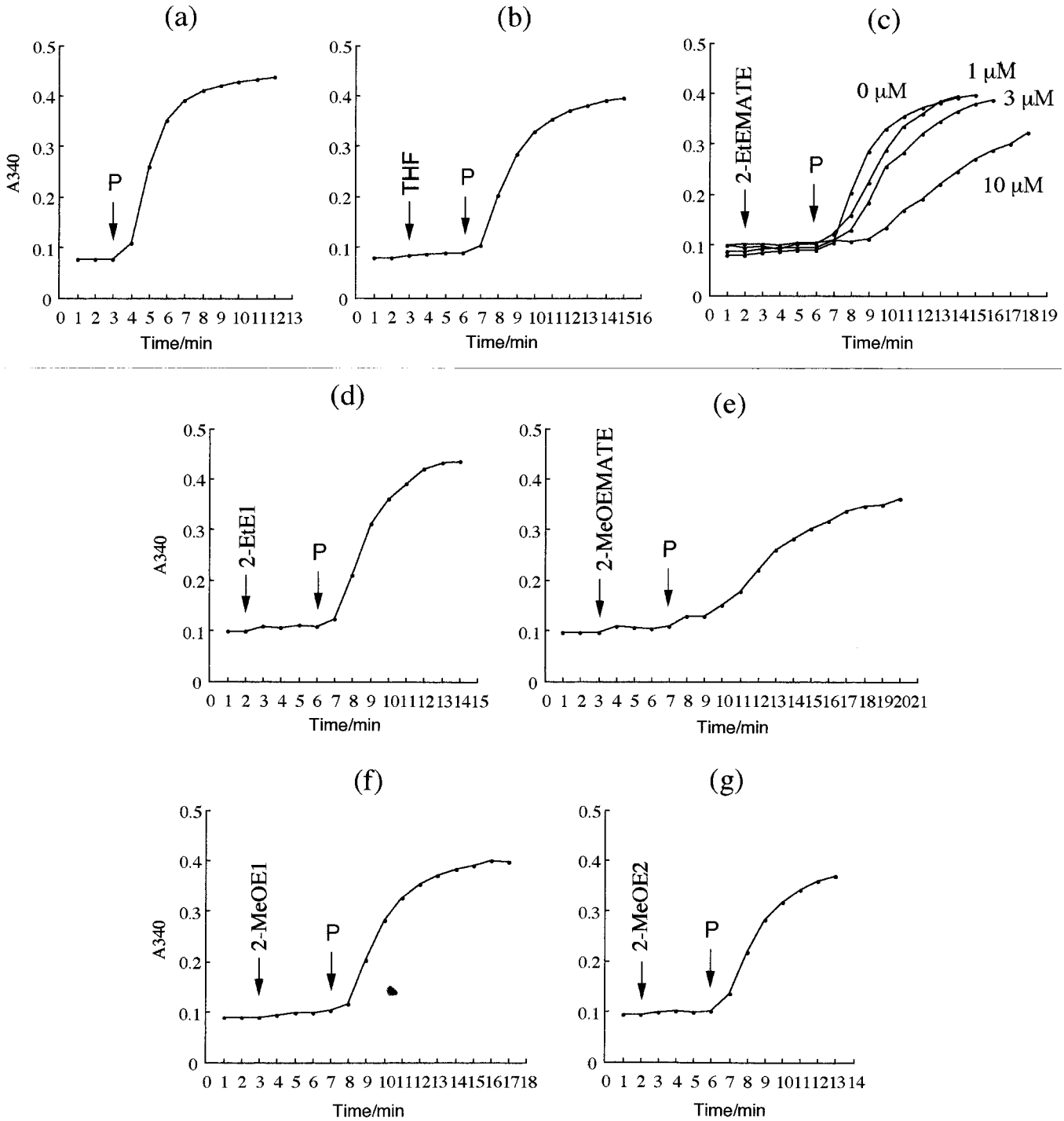


Fig. 8. Tubulin turbidimetry. *In vitro* tubulin assembly was measured by turbidity. Drugs (*P*, paclitaxel) were added directly to the cuvette as indicated (all at 10 μM, unless otherwise indicated). Results presented are representative of three experiments.

pearance of slower migrating isoforms in immunoblotting experiments) was clearly induced by 2-EtEMATE and paclitaxel, but not by 2-EtE1, in MCF7, CAL51, and CAMA1 cells (Fig. 10). The concentrations that induced these modifications were similar to those that inhibited cell growth (Fig. 2). Similar results were obtained for MCF7 cells treated with 2-EtEMATE for both shorter and longer times and also for 2-MeOEMATE (data not shown). There was little evidence for phosphorylation of BCL-2 or BCL-X_L induced by either of the estrone derivatives in ZR-75-1, which was the most resistant line tested. Therefore, similar to other antimicrotubule agents, the sulfamoylated estrone derivatives induce phosphorylation of antiapoptotic BCL-2 family proteins in at least some breast cancer cell lines.

p53 was significantly induced in CAL51 cells and to a lesser extent in MCF7 cells (which are known to express wild-type p53) treated with paclitaxel or 2-EtEMATE but not with 2-EtE1 (Fig. 10). Similar to phosphorylation of BCL-2 and BCL-X_L, the concentrations of drugs required to increase p53 expression closely matched those that inhibited cell growth. p53 expression was relatively low in ZR-75-1 cells and was not significantly affected by any of the drugs tested. By contrast, p53 levels were very high in control CAMA1 cells, which

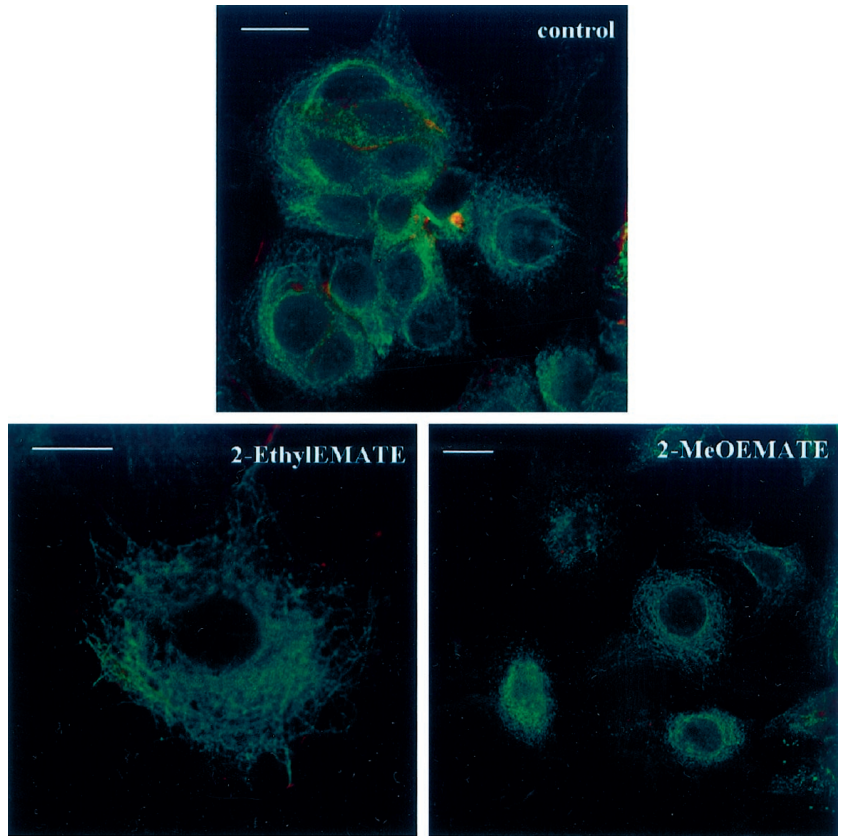


Fig. 9. Immunofluorescence analysis of microtubule structures in MCF7 cells. MCF7 cells were grown on collagen-coated glass slides in the presence of the indicated drugs (5 μM) for 16 h or left untreated as a control. Cells were fixed and analyzed using a β-tubulin-specific antibody. The *bar* in the *top left-hand corner* of each image is equivalent to 20 μm. Results presented are representative of three experiments.

suggests stabilizing mutation, and this expression was not altered by any of the compounds (Fig. 10). Because CAMA1 cells are relatively sensitive to 2-EtEMATE, the induction of p53 is unlikely to be essential for its effects on cell cycle or apoptosis in breast cancer cells.

DISCUSSION

We demonstrated previously (21) that 2-MeOEMATE, but not the nonsulfamoylated parent estrone 2-MeOE1, induced a G_2-M arrest and modest apoptosis in MCF7 cells *in vitro* and the regression of nitrosomethylurea-induced breast tumors in rats. This suggested that, like the endogenous estradiol metabolite 2-MeOE2, certain estrone derivatives might be useful as novel agents for the treatment and prevention of breast cancer, and that sulfamoylation may be an effective mechanism for enhancing their anticancer activity. The aims of the experiments presented here were to explore further the significance of the sulfamate modification, to identify additional conjugated estrone derivatives with anticancer activity, and to understand their mechanism of action.

A consistent result obtained from these studies is that sulfamoylation potently enhances the anticancer activity of the estrone derivatives. The sulfamoylated compounds were >10-fold more potent than the parent compounds in growth assays (long- and short-term) in the induction of phosphorylation of BCL-2 and BCL-X_L and p53 expression and in the inhibition of paclitaxel-driven *in vitro* tubulin assembly. The sulfamoylated compounds were also irreversible inhibitors of cell growth. Results from *in vitro* tubulin assembly assays suggest that the sulfamoylated compounds have an increased affinity for tubulin itself, and this may underlie the enhanced activity of these compounds. Whether this reflects enhanced binding specifically for tubulin or an increased affinity for proteins generally, and whether this mediates sequestration of EMATE in RBCs and can fully account for the enhanced biological activity of the sulfamoylated compounds, remains to be determined. It was also evident from our study that 2-MeOEMATE was a more potent inhibitor of cell growth than 2-MeOE2, which has received much interest as an endogenous estrogen with antimitotic properties (2). In contrast to 2-MeOEMATE, 2-MeOE2 was a reversible inhibitor of cell growth and did not inhibit tubulin assembly *in vitro* at the concentration tested in our assay. It will be interesting to determine whether the sulfamoylated estrones possess antiangiogenic properties, similar to 2-MeOE2, and whether sulfamoylation would enhance the growth-inhibitory activity of 2-MeOE2.

The ability of the sulfamoylated estrone derivatives to induce a mitotic arrest and apoptosis is most likely attributable to their antimicrotubule activity. 2-EtEMATE and 2-MeOEMATE inhibited the ability of paclitaxel to promote tubulin assembly *in vitro*. Therefore, the sulfamoylated estrone derivatives seem to share many features of their activity with compounds described as microtubule-destabilizing agents such as colchicine and the *Vinca* alkaloids, vincristine and vinblastine. Consistent with this, 2-MeOE2 binds to β-tubulin at the same site as colchicine and also inhibits tubulin assembly (10, 22). 2-EtE2 was also reported to be an antimicrotubule agent (22). As discussed above, it is possible that sulfamoylation increases the affinity of these compounds for tubulin. Alternatively, the modification may facilitate binding to β-tubulin at different/additional sites, or it may even engender an irreversible covalent modification as proposed for the interaction of steroid sulfamates with steroid sulfatase (28). Our results are not consistent with previous reports suggesting that, like paclitaxel, 2-MeOE2 increases tubulin mass in intact cells (13). However, it should be noted that the amount of insoluble tubulin in drug-treated cells seemed to be very small in this study. It is apparent, however, from our analysis of microtubule structures in drug-treated

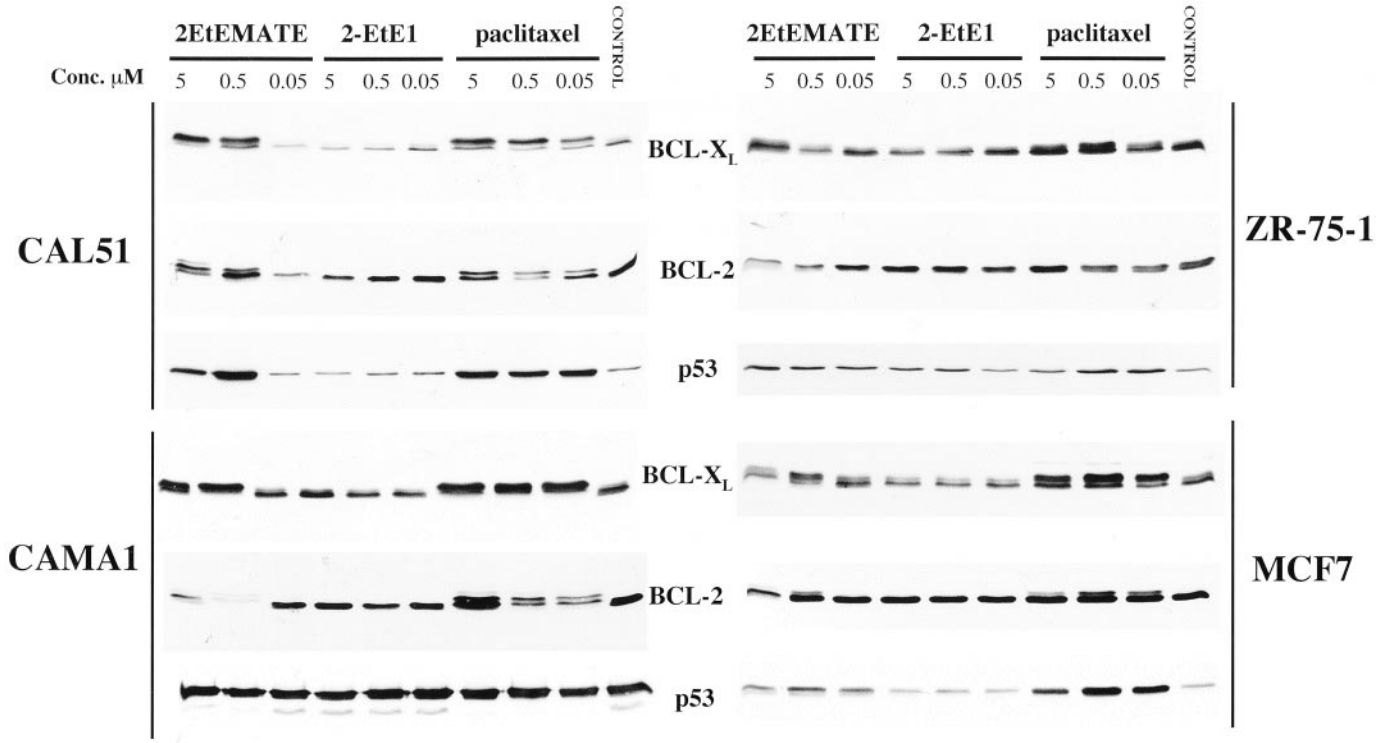


Fig. 10. Expression of BCL-2, BCL-X_L, and p53 in breast cancer cell lines. MCF7, ZR-75-1, CAL51, and CAMA1 cells were exposed to the indicated concentrations of 2-EtEMATE, 2-EtE1, or paclitaxel for 24 h or left untreated as a control. Expression of BCL-2, BCL-X_L, and p53 were determined by immunoblotting. Note that each panel represents a different exposure time. The relative expression of BCL-X_L and BCL-2 in the cell lines is indicated in the text. Results presented are representative of at least three experiments.

cells that although the sulfamoylated estrone derivatives target tubulin directly, the cell cycle inhibitory effects of the sulfamoylated estrone derivatives are not associated with gross changes in microtubule structures in interphase cells. This is consistent with an emerging picture that shows that the fundamental activity of many antimicrotubule agents at physiologically relevant concentrations is on microtubule dynamics, rather than directly on tubulin polymerization (14). We therefore suggest that the sulfamoylated estrone derivatives function as effective inhibitors of microtubule assembly, but that their antimitotic activity stems from the inhibition of microtubule dynamics rather than from the wholesale disassembly of the microtubule network.

The sulfamoylated estrone derivatives were potent inducers of apoptosis in some cell lines, and our comparison sheds light on their likely mechanism of apoptosis. Although, like paclitaxel, 2-EtEMATE induced p53 expression in MCF7 and CAL51 cells, it is unlikely that induction of p53 function is essential for cell death/cell cycle arrest. CAMA1 cells were relatively sensitive to apoptosis but expressed high levels of p53, a hallmark of inactivating mutation, which was not regulated by the compounds.

Our results also demonstrate that the effects of the sulfamoylated estrone derivatives are not dependent on the ER. CAL51 cells lack ERα protein expression (23) but are highly sensitive to growth inhibition, confirming our previous observations using ER-negative MDA-MB-231 cells (21). Consistent with this finding, we were unable to detect ERα protein expression in CAMA1 cells that were also very sensitive to the sulfamoylated estrones (24). However, the significance of this is unclear, inasmuch as CAMA1 cells have been shown previously to express ERs (29). It is possible that the presence of estrogens in our growth conditions might account for this potential discrepancy, because estrogens have been shown to down-regulate ER expression (30). It was also clear that ER function did not play a major role in relative resistance to the compounds in ER-positive MCF7 cells, because inhibition of ER function by ICI 182,780 did not modulate growth inhibition.

In contrast to p53, expression of the antiapoptotic BCL-2 protein may be an important determinant of sensitivity. MCF7 and ZR-75-1 cells expressed high levels of BCL-2 (~50-fold higher than CAL51 and CAMA1 cells) and were relatively resistant to cell killing. Thus, BCL-2 expression correlates positively with resistance to cell killing. The apoptosis of MCF7 cells that occurs at high concentrations (10 μM; Ref. 21) is likely to involve inactivation of BCL-2 by phosphorylation (by preventing binding to BAX). Therefore, it is surprising, perhaps, that ICI 182,780 did not sensitize MCF7 cells to the sulfamoylated estrone derivatives, because inhibition of ER function leads to down-regulation of BCL-2 expression (31, 32). However, ICI 182,780 is likely to have many effects on MCF7 cells in addition to BCL-2 regulation, and additional experiments are required to test the significance of BCL-2 expression and phosphorylation. Although we also readily detected phosphorylation of BCL-X_L in breast cancer cells, its expression was more consistent in the breast cancer cells studied and would not, therefore, be expected to contribute significantly to differential sensitivity. However, it is worthwhile to note that Attalla *et al.* (13) showed previously that BCL-X_L was not phosphorylated in K562 cells treated with 2-MeOE2. It will be interesting to determine whether this difference reflects a cell-specific effect or whether true differences exist in the ability of estrogen derivatives to activate the kinases involved in phosphorylation of antiapoptotic BCL-2-family proteins, which might account for enhanced cell killing.

The present study not only provides significant new rationalization for the potent effects of 2-MeOEMATE but also demonstrates that similar effects can be achieved using the sulfamoylated derivative of an unnatural estrogen derivative, 2-EtE1. This synthetic derivative is isosteric with 2-MeOE1, and the results, therefore, indicate that the 2-position oxygen atom of 2-MeOEMATE does not seem to play a

significant role in the activity of this compound. By contrast, a much higher concentration (>20 μM) of a related derivative, 2-propyl-EMATE (20), was required to affect cell growth and morphology (data not shown). This shows that chain extension of the 2-ethyl derivative attenuates its activity, presumably by exclusion from a binding pocket. Similar conclusions were reached by Cushman *et al.* (22) in a study on the antitubulin activity of a number of 2-modified estradiol analogues, including 2-EtE2. Clearly, additional detailed structure activity studies focusing on the interplay of the 2-position substituent and the sulfamate group are warranted, and these are in progress.

The potent and irreversible *in vitro* growth-inhibitory effects and antimicrotubule activity of the sulfamoylated estrone derivatives make them attractive potential agents for breast cancer treatment and prevention: (*a*) the drugs are available for administration p.o. and relatively easy and inexpensive to synthesize; and (*b*) oral administration of estrogens, including 2-MeOEMATE in rats (21), does not seem to be associated with significant toxicities. Most important, sulfamoylation seems to be a useful approach to enhance the biological effects of these compounds. Sequestration into RBCs may provide an *in vivo* reservoir protecting the compounds from clearance or inactivation. Detailed animal experiments are warranted to explore further the potential use of sulfamoylated estrone derivatives as anticancer agents.

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Differential Effects of Estrone and Estrone-3-*O*-Sulfamate Derivatives on Mitotic Arrest, Apoptosis, and Microtubule Assembly in Human Breast Cancer Cells

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