Microsomal antiestrogen-binding site ligands induce growth control and differentiation of human breast cancer cells through the modulation of cholesterol metabolism

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Abstract

The microsomal antiestrogen-binding site (AEBS) is a high-affinity membranous binding site for the antitumor drug tamoxifen that selectively binds diphenylmethane derivatives of tamoxifen such as PBPE and mediates their antiproliferative properties. The AEBS is a hetero-oligomeric complex consisting of 3β-hydroxysterol-Δ^8-Δ^7-isomerase and 3β-hydroxysterol-Δ^7-reductase. High-affinity AEBS ligands inhibit these enzymes leading to the massive intracellular accumulation of zymostenol or 7-dehydrocholesterol (DHC), thus linking AEBS binding to the modulation of cholesterol metabolism and growth control. The aim of the present study was to gain more insight into the control of breast cancer cell growth by AEBS ligands. We report that PBPE and tamoxifen treatment induced differentiation in human breast adenocarcinoma cells MCF-7 as indicated by the arrest of cells in the G_0-G_1 phase of the cell cycle, the increase in the cell volume, the accumulation and secretion of lipids, and a milk fat globule protein found in milk. These effects were observed with other AEBS ligands and with zymostenol and DHC. Vitamin E abrogates the induction of differentiation and reverses the control of cell growth produced by AEBS ligands, zymostenol, and DHC, showing the importance of the oxidative processes in this effect. AEBS ligands induced differentiation in estrogen receptor-negative mammary tumor cell lines SKBr-3 and MDA-MB-468 but with a lower efficiency than observed with MCF-7. Together, these data show that AEBS ligands exert an antiproliferative effect on mammary cancer cells by inducing cell differentiation and growth arrest and highlight the importance of cholesterol metabolism in these effects. [Mol Cancer Ther 2008;7(12):3707–18]

Introduction

The microsomal antiestrogen-binding site (AEBS) was first described in the 1980s as a high-affinity binding site for tamoxifen, distinct from the estrogen receptors (ER; ref. 1). Structure-affinity studies revealed that the AEBS, as opposed to the ER, does not bind estrogens and antiestrogens devoid of a protonable amino-ethoxy side chain but binds selective ER modulators (SERM) such as tamoxifen and raloxifene (2, 3). The identification of diphenylmethane derivatives of tamoxifen that selectively target the AEBS, such as PBPE and DPPE, enabled the functions and pharmacology of the AEBS to be studied (4, 5). As opposed to tamoxifen, diphenylmethane compounds have no affinity for the ER. Moreover, PBPE and DPPE are not inhibitors of other known targets for tamoxifen such as protein kinase C, calmodulin, or acyl-coenzyme A:cholesterol acyltransferase activities (3, 6). We and others have reported that the AEBS may account for the antiproliferative and cytotoxic activity of its cognate ligands, but there has been little work done on the nature of the growth control induced by these ligands (7, 8).

Earlier studies reported that tamoxifen induced the accumulation of zymostenol in the blood of patients (9). These data, coupled with the fact that the AEBS bound oxygenated derivatives of sterols such as 7-ketocholesterol, 6-ketocholestanol, and 7-ketocholestanol with high affinity (10, 11), opened up the possibility of a link between the binding to the AEBS and the oxidative metabolism of cholesterol. We confirmed this hypothesis by showing that AEBS ligands induced a major modification of cholesterol metabolism in tumor cells and afforded the molecular identification of the AEBS (2). We showed that, when tumor cells were exposed to tamoxifen and PBPE at concentrations that induced growth control, the neosynthesis of cholesterol was stopped and cells accumulated

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cholesterol precursors that were not found in cells before the treatments. The major metabolites identified in human breast adenocarcinoma MCF-7 cells were 3β-hydroxyl-cholest-8-ene (zymostenol) for tamoxifen and PBPE treatment and 3β-hydroxyl-cholesta-5,7-diene [7-dehydrocholesterol (DHC)] for PBPE treatment. We showed that this accumulation was due to a noncompetitive inhibition of the two enzymes, the 3β-hydroxysterol-Δ^8-Δ^7-isomerase (D8D7I) and the 3β-hydroxysterol-Δ^7-reductase (DHCR-7) that use zymostenol and DHC, respectively, as substrate. The coexpression of the D8D7I and DHCR-7 was found necessary and sufficient for the reconstitution of the AEBS in mammalian cells, indicating that the AEBS consisted of both enzymes (2).

The aim of the present study was to probe further the nature of the growth control mediated by AEBS ligands on breast cancer cells. We evaluated the effect of the selective AEBS ligand PBPE, tamoxifen, and sterols that accumulate under AEBS ligand treatments such as zymostenol and DHC on the control of growth and phenotypic modification of cells.

Materials and Methods

Chemicals and Antibodies

PBPE, DPPE, and MBPE were synthesized in our laboratory as described previously (5). Zymostenol was purified as described before and was 99% pure by high-performance liquid chromatography (2). Commercial sterols were purified by high-performance liquid chromatography and stored under argon before use. Other compounds and chemicals were from Sigma-Aldrich. All solvents were from Carlo Erba. Monoclonal anti-human milk fat globulin (MFG) antibody was from Chemicon (ABA4087, clone ICO-103). Anti-glyceraldehyde 3-phosphate dehydrogenase antibodies were from Santa Cruz Biotechnology, and secondary antibodies were from Jackson Immunoresearch.

Cell Culture

Breast cancer cell lines were from the American Type Culture Collection and cultured until passage 30. Cells were maintained in RPMI 1640 supplemented with 2 g/L sodium bicarbonate, 1.2 mmol/L glutamine (pH 7.4), 5% fetal bovine serum for MCF-7 cells, ZR75-1 and TSA or 10% fetal bovine serum for other cell lines, and both penicillin and streptomycin (50 units/mL) in a humidified atmosphere with 5% CO_2 at 37°C. MCF-7^{ws} were grown as described before (2). For the measurement of the antiproliferative indices, 10^5 cells were plated onto 6-well plates and treated for 48 h after plating with 10, 15, and 20 μmol/L PBPE or 1, 2.5, 5, or 10 μmol/L tamoxifen or the solvent vehicle (0.1% ethanol) for 5 days. Drugs and medium were changed every 48 h. Cells were counted daily. Experiments were repeated in triplicates. Dose curves were plotted as a function of cell number versus time. Fifty percent maximal effective concentration (EC_{50}) values were calculated using Prism software (GraphPad Software).

Cell Cycle Analysis

Cells were washed with PBS, fixed in ice-cold absolute ethanol for 30 min at 4°C, and washed twice again in PBS. Cell cycle was analyzed by fluorescence-activated cell sorting (FACS) flow analysis exactly as described before (12), with data were obtained from 10^5 viable cells.

Oil Red O Staining Procedure

Cells were grown on glass coverslips and treated with drugs for 72 h (3 days) and then fixed with 3.7% paraformaldehyde for 1 h at room temperature followed by washing twice with PBS (Euromedex). Oil red O (ORO) staining and quantification of lipids were done according to published procedure (13).

Lipid Analyses

MCF-7 cells (5 million) were treated for 48 h with the solvent vehicle, 10 μmol/L PBPE, or 2.5 μmol/L tamoxifen. Cells were then washed three times in PBS, and lipids were extracted by liquid extraction according to the method of Bligh and Dyer (14). Neutral lipids and sphingomyelin analyses were done according to previously published procedures (15, 16). For secreted lipids, MCF-7^{ws} cells were treated for 48 h with the solvent vehicle, 2.5 μmol/L PBPE, or 1 μmol/L tamoxifen. The culture medium (50 mL) was centrifuged for 10 min at 1,000 rpm. The supernatant was reduced to 100 μL by lyophilization and lipids were extracted and analyzed as described above.

Transmission Electron Microscopy

Cells were fixed with 2% glutaraldehyde in 0.1 mol/L Sorensen phosphate buffer (pH 7.4) for 1 h and washed with the Sorensen phosphate buffer (0.1 mol/L) for 12 h. The cells were then postfixed with 1% OsO_4 in Sorensen phosphate buffer (0.05 mol/L Sorensen phosphate buffer, 0.25 mol/L glucose, 1% OsO_4) for 1 h. The cells were then washed twice with distilled water and prestained with an aqueous solution of 2% uranyl acetate for 12 h. Samples were then treated exactly as described previously (6).

Immunocytochemistry

Cultured cells were fixed with a methanol/acetone solution (1:1) for 15 s. Cells were washed twice with distilled water and incubated for 30 min at 37°C with a solution of RNase A (10 μg/mL in PBS). Then, the cells were incubated for 1 h with propidium iodide (2 μg/mL in PBS). The slides were then washed with PBS and blocked with 5% bovine serum albumin in PBS. Cells were incubated for 1 h with the primary antibody (mouse anti-human MFG, 1:100) at 37°C. The slides were then washed three times with 1% bovine serum albumin in PBS and incubated with the anti-mouse fluorescein-conjugated antibody (1:100 in 5% bovine serum albumin in PBS) for 1 h at 37°C. The slides were then washed with PBS and mounted with Moviol 4-88 reagent (Calbiochem). Cells were viewed and images were captured using a confocal microscope Zeiss LSM 510 inverted microscope with a plan-apochromat ×63/1.20 oil immersion objective. Images were prepared using a Zeiss LSM Image Viewer.

AEBS Binding Assay

Binding assays were done exactly according to a previously published procedure (17).

Western Blotting

Immunoblotting was carried out as described previously (2). For the detection of MFG, proteins were separated on a 10% SDS-PAGE gels, electrotransferred onto polyvinylidene difluoride membranes, and incubated overnight at 4°C with the mouse anti-human MFG (1:1,000) or the mouse anti-human glyceraldehyde 3-phosphate dehydrogenase (1:1,000). Visualization was achieved with an Enhanced Chemiluminescence Plus kit (Amersham Biosciences) and fluorescence was measured by either autoradiography or using a PhosphorImager (Storm 840; Amersham Biosciences). Analyses of secreted MFG were carried out using conditioned medium from MCF-7 cells cultured up to 3 days in the presence or absence of 10 μmol/L PBPE or 2.5 μmol/L tamoxifen. Samples (400 μL) were loaded into individual wells of the slot blot apparatus (Hoefer) and transferred by vacuum onto a polyvinylidene difluoride membrane pre-wetted with transfer buffer and saturated with saline. The membranes were treated for MFG revelation as described above.

Proliferation Assays

MCF-7 cells were seeded in RPMI 1640 with 5% FCS into 12-well plates at 30,000 per well. The cells were then treated for 3 days with 10 μmol/L PBPE or 2.5 μmol/L tamoxifen in the presence or absence of 500 μmol/L vitamin E for 3 days. Drugs and medium were changed after 48 h. Cells were harvested by trypsinization and counted on a Coulter counter. Experiments were repeated in triplicate.

Biostatistical Analysis

Values are mean ± SE of three independent experiments each carried out in duplicate. Statistical analysis was carried out using a Student's t test for unpaired variables. ★ and ★★ in the figures refer to statistical probabilities (P) of <0.001 and <0.0001, respectively, compared with control cells that received the solvent vehicle alone.

Results

AEBS Ligands Induce Breast Cancer Cell Differentiation

To gain more insights into the antiproliferative action of AEBS ligands, we have carried out cell cycle and morphologic studies on MCF-7 cells treated with the selective AEBS ligand PBPE or with tamoxifen. There was a concentration- and time-dependent inhibition of cell proliferation with PBPE and tamoxifen, with a cytostatic effect observable with 10 μmol/L PBPE and 2.5 μmol/L tamoxifen (Fig. 1A). Three-day treatment of MCF-7 cells with 10 μmol/L PBPE and 2.5 μmol/L tamoxifen caused 76 ± 1% and 74 ± 1% arrest in the G_0-G_1 phase of the cell cycle (Fig. 1B), respectively, compared with 43.6 ± 2% for cells treated with the vehicle. The G_0-G_1-S ratios were 4.8 and 4.4 times higher for PBPE and tamoxifen, respectively, compared with the control. This effect was associated with drastic changes in the cell morphology (Fig. 1C). PBPE- and tamoxifen-treated cells increased in size and flattened, which is a characteristic of MCF-7 cell differentiation (18). The increase in size was attributable to a decrease in the nucleus/cytoplasm ratio. We then stained the cells with ORO that colored red the neutral lipids such as triacylglycerol, free sterols, and fatty acid-esterified sterols. ORO staining of MCF-7 cells revealed that PBPE and tamoxifen increased the number of cells containing lipid droplets from 25 ± 5% to 75 ± 6% and 73 ± 6%, respectively (Fig. 1C). We observed a time- and concentration-dependent accumulation of lipid droplets with PBPE (Supplementary Fig. S1A)[7] and tamoxifen (Supplementary Fig. S1B).[7] Lipid droplet appearance occurred at different cell densities (Supplementary Fig. S2)[7] and is not dependent on cell density.

Ultrastructure analyses by electron microscopy of MCF-7 cells treated with PBPE and tamoxifen completed these experiments. As shown in Fig. 1D (*1*), MCF-7 cells treated over 3 days with 10 μmol/L PBPE contained both unilamellar vesicles (Fig. 1D, 2) and multilamellar bodies (Fig. 1D, 3). We noted that multilamellar bodies were observed after only a few hours of treatment, whereas unilamellar vesicles were detected after a lag time of 12 h. Identical ultrastructural changes were observed after treatment with 2.5 μmol/L tamoxifen, but the abundance of unilamellar vesicles was half that obtained with PBPE (data not shown).

Unilamellar vesicles are found in lactating cells and have been characterized as vesicles for the storage and secretion of triacylglycerol (19). Multilamellar bodies have been characterized as storage and secretion structures that contain sterols and phospholipids (20). Thus, both vesicle types containing neutral lipids can be colored with ORO and can account for the positive coloration obtained with ORO after treatment with PBPE or tamoxifen. We therefore analyzed the nature of lipids that accumulated after PBPE and tamoxifen treatment because the morphologic and biochemical changes produced by the test compounds were suggestive of epithelial mammary differentiation.

AEBS Ligands Induce the Production and Secretion of Lipids Found in Milk in MCF-7 Cells

Lipids overproduced by differentiated mammary epithelial cells are complex mixtures containing predominantly triacylglycerol as well as sterols and phospholipids such as sphingolipids (21). To determine whether these lipids were accumulated after PBPE or tamoxifen treatments, MCF-7 cells were treated for 3 days with 10 μmol/L PBPE or 2.5 μmol/L tamoxifen and collected, and the extracted lipids were analyzed by gas-liquid chromatography. The results from the quantification of sterols are shown on Fig. 2A. The results showed that PBPE and tamoxifen treatment enhanced the intracellular content of free sterols by 2.17- and 2.01-fold, respectively, compared with control cells (Fig. 2A). These increases in total sterols explain the appearance of multilamellar bodies in cells (Fig. 1C). In addition, we showed that PBPE and tamoxifen increased the intracellular level of triacylglycerol by 4.44- and

[7] Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

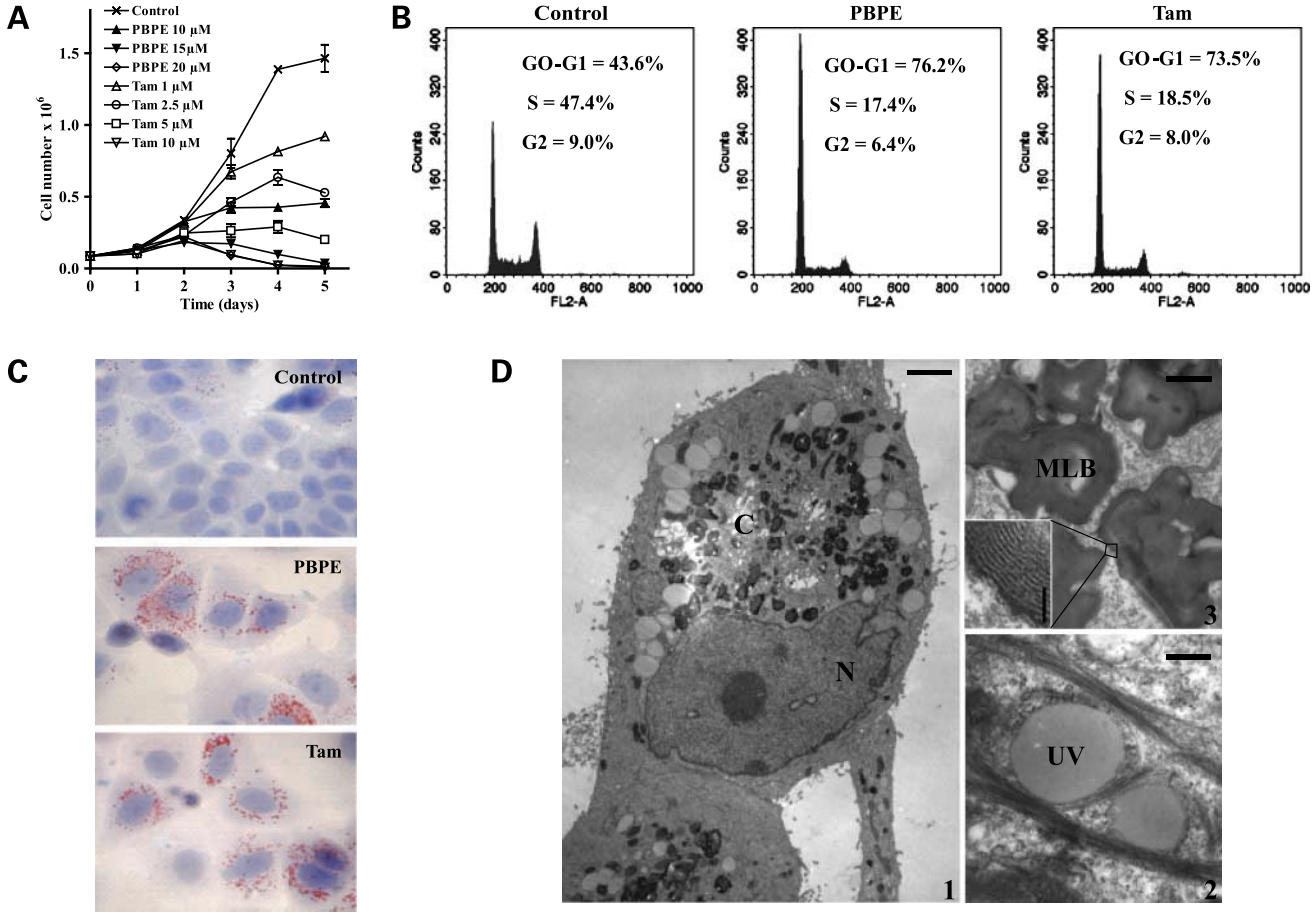


Figure 1. PBPE and tamoxifen induce growth control and characteristics of differentiation in MCF-7 cells. **A,** measurement of the antiproliferative index. MCF-7 cells (10^5) were plated into 6-well plates and treated for 48 h after plating with 10, 15, and 20 μmol/L PBPE or 1, 2.5, 5, and 10 μmol/L tamoxifen or the solvent vehicle (0.1% ethanol) for 5 d. Drugs and medium were changed every 48 h. Cells were counted daily. Cells were harvested by trypsinization and counted on a Coulter counter. Experiments were repeated in triplicates. Dose curves were plotted as a function of cell number versus time. **B,** cell cycle distribution of MCF-7 cells treated with solvent vehicle (*Control*), 10 μmol/L PBPE, or 2.5 μmol/L tamoxifen for 3 d. Cell cycle distribution was measured as described in Materials and Methods by FACS flow analysis using the Becton Dickinson FACS system. **C,** staining of neutral lipids with ORO in MCF-7 cells treated with solvent vehicle, 10 μmol/L PBPE, or 2.5 μmol/L tamoxifen for 3 d. Morphologic and biochemical changes were evaluated by light microscopy (×40) of ORO-stained cells counterstained with Mayer's hematoxylin as described in Materials and Methods. **D,** electron micrographs of MCF-7 cells treated with 10 μmol/L PBPE (*1*) for 3 d. After fixation of the cells and embedding in Epon 812, ultrathin sections of the cells were prepared, stained with uranyl acetate and lead citrate, and examined in a H300 Hitachi electron microscope. Two types of vesicles are found in cells treated with PBPE: unilamellar vesicles (*UV*; *2*) and multilamellar bodies (*MLB*; *3*). *N,* nucleus; *C,* cytoplasm. *Bars,* 6.6 μm for *1*, 0.33 μm for *2* and *3* and 33 nm for *3* (*inset*).

2.42-fold, respectively, compared with the control cells. The accumulation of triacylglycerol explains the presence of unilamellar vesicles in the cytoplasm of the treated cells (Fig. 1D). Kinetics studies done over 2 days of treatment with PBPE or tamoxifen showed that sterols started accumulating a few hours after the beginning of the treatment and reached a plateau after 24 h, with a half saturation time of accumulation of 6 h. The increase in the triacylglycerol content was visible at 12 h and plateaued after 24 h of treatment with a half saturation time of accumulation of 18 h. This shows a sequential accumulation of neutral lipids beginning with sterols and followed by triacylglycerol. PBPE and tamoxifen also induced a 2.56- and 2.62-fold increase in sphingomyelin in MCF-7 cells compared with the controls. Sphingomyelin is a phospholipid reported to be important for the formation of vesicles (20) and is one of the major phospholipids found in milk (ref. 21; Fig. 2C). Together, these data indicate that PBPE and tamoxifen stimulated the accumulation of lipid species found in milk during lactation and are consistent with mammary epithelial cell differentiation.

The next question we addressed was whether the lipids detected in treated cells were also secreted into the culture medium. To answer this question, we used MCF-7 cells (MCF-7^{ws}) adapted to grow in a chemically defined medium without serum to avoid contamination with serum lipids as reported previously (2). We showed that MCF-7^{ws} cells reacted in the same way as MCF-7 cells to PBPE or tamoxifen treatments, although MCF-7^{ws} cells were more sensitive to AEBS ligands than MCF-7 cells. To avoid a

contamination of the culture medium due to cytolysis, we chose a lower concentration in PBPE and tamoxifen than that used with MCF-7 cells. Indeed, 2-day treatment of MCF-7^{ws} cells with 2.5 μmol/L PBPE or 1 μmol/L tamoxifen were sufficient to induce, respectively, 75 ± 2% and 72 ± 2% accumulation of cells in the G_0-G_1 phase of the cell cycle against 56 ± 2% for the control cells as was measured for the MCF-7 cells. The MCF-7^{ws} cells treated with PBPE and tamoxifen were 75 ± 8% and 78 ± 7% ORO positive, respectively, compared with 18 ± 2% in control cells (data not shown). The toxicity was not significantly different between treated cells and control cells and was <1%. The accumulated sterols and triacylglycerol were found in the same proportions as in MCF-7 cells (ref. 2; data not shown). The qualitative and quantitative analyses by gas-liquid chromatography of the lipids extracted from the culture medium showed that 2-day treatment of MCF-7^{ws} cells with 2.5 μmol/L PBPE or 1 μmol/L tamoxifen increased the amount of sphingomyelin, sterols, and triacylglycerol in the culture medium by 9- to 14-fold compared with the controls (Fig. 2D). These data indicated that the lipids accumulated within the cells as a result of PBPE and tamoxifen treatment were actively secreted into the culture medium. Altogether, these data showed that AEBS ligands induced the accumulation and the secretion of the different classes of lipids found in milk.

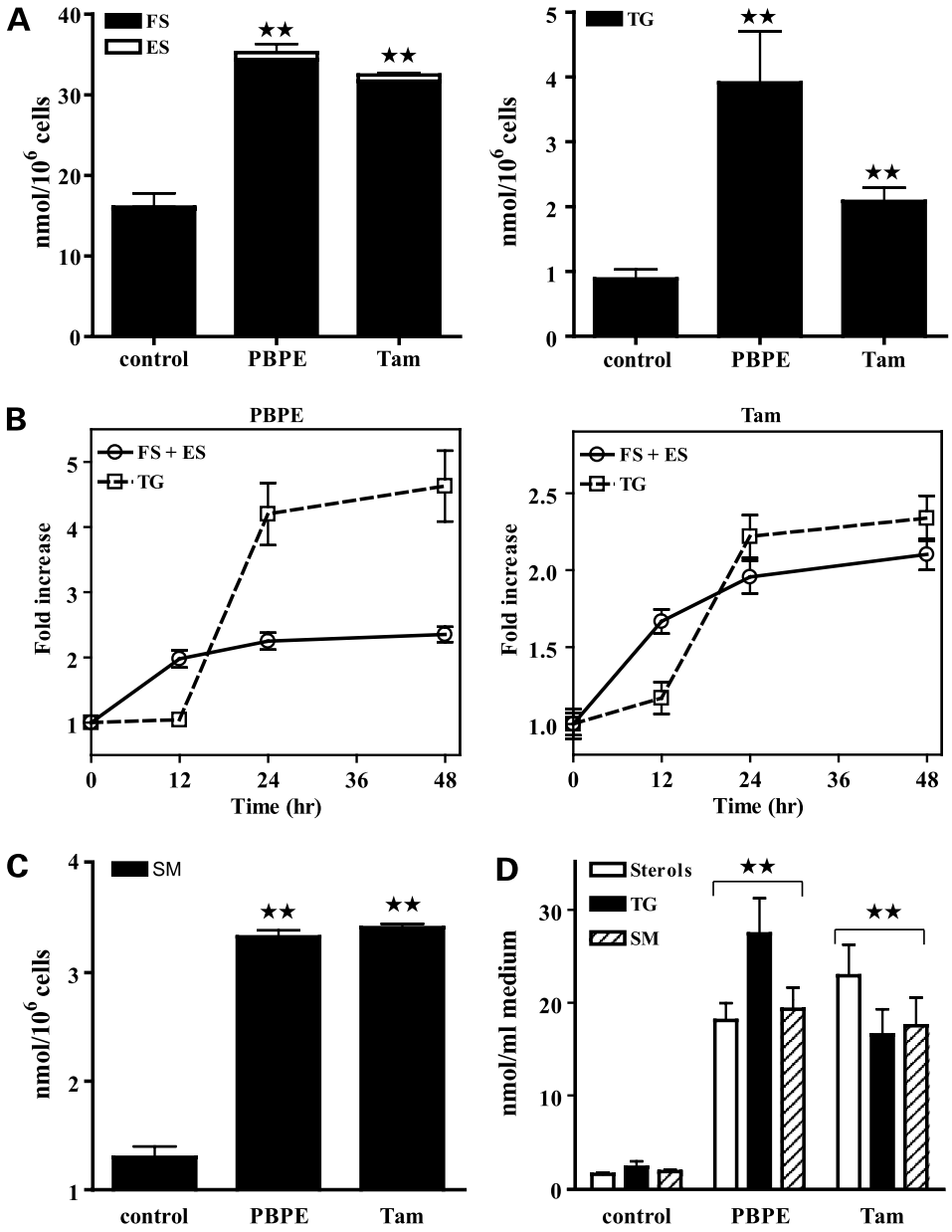


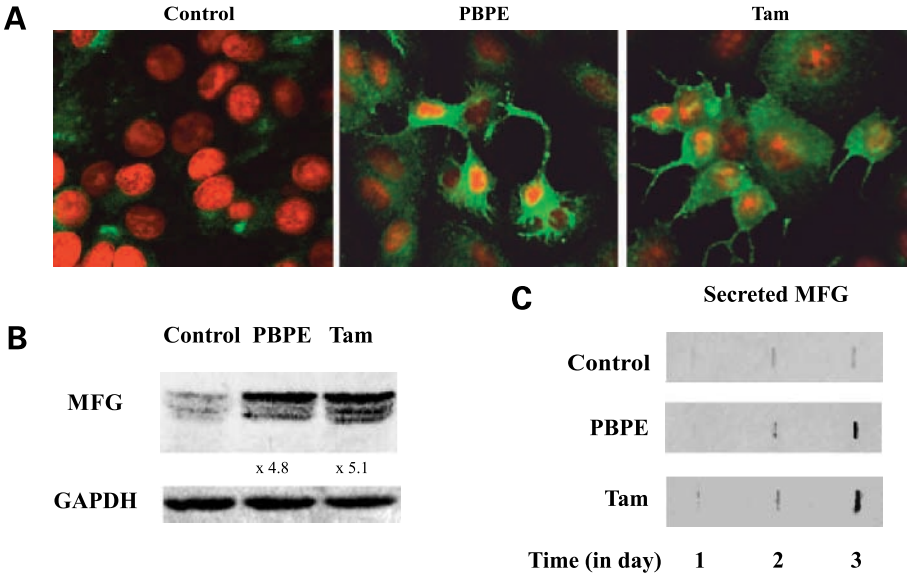
Figure 2. PBPE and tamoxifen stimulate the accumulation and secretion of lipids by MCF-7 cells. **A,** characterization and quantification of free sterol (*FS*) and esterified sterols (*ES*) and triacylglycerols (*TG*) present in MCF-7 cells were carried out as described in Materials and Methods. MCF-7 cells were treated with solvent vehicle, 10 μmol/L PBPE, or 2.5 μmol/L tamoxifen for 3 d. **B,** kinetic studies of the accumulation of free sterols and esterified sterols (○) and triacylglycerols (□) in MCF-7 cells treated with 10 μmol/L PBPE and 2.5 μmol/L tamoxifen for 0, 12, 24, and 48 h. **C,** quantification of sphingomyelin in MCF-7 cells treated with solvent vehicle, 10 μmol/L PBPE, or 2.5 μmol/L tamoxifen for 3 d was done as described in Materials and Methods. **D,** lipid content analysis of the culture medium of MCF-7^{ws} cells treated with the solvent vehicle, 2.5 μmol/L PBPE, or 1 μmol/L tamoxifen for 3 d was carried out as described in Materials and Methods. MCF-7^{ws} are MCF-7 cells adapted to grow in a chemically defined medium in the absence of serum and thus devoid of exogenous lipids and proteins. Mean ± SE of three independent experiments. ★, $P < 0.001$, compared with control; ★★, $P < 0.0001$, compared with control.

AEBS Ligands Induce the Expression and Secretion of MFG by MCF-7 Cells

Mammary epithelial cells of lactating mammary glands produce and secrete fat droplets and proteins such as MFG that are associated with lipid droplets (22). Thus, in addition to lipids found in milk, we investigated the modulation of the expression and secretion of MFG in MCF-7 cells treated with PBPE or tamoxifen. Immunocytochemical analysis showed that 10 μmol/L PBPE and 2.5 μmol/L tamoxifen stimulated the expression of MFG that were not detectable in the control cells (Fig. 3A). Western blot analyses of cell proteins confirmed these results and revealed that PBPE and tamoxifen induced a considerable increase in the expression of MFG proteins compared with the control cells (Fig. 3B). Dot-blot analysis of the proteins present in the culture medium showed that 2 and 3 days treatment of cells with PBPE and tamoxifen increased the secretion of MFG into the medium compared with the control cells (Fig. 3C). These data showed that AEBS ligands stimulate the expression and the secretion of MFG by MCF-7 cells as observed in lactating epithelial cells (22).

AEBS Ligands Belonging to Different Structural Classes Induce Differentiation Characteristics in MCF-7 Cells

We next tested different categories of AEBS ligands to determine whether they induced differentiation characteristics in MCF-7 cells (Fig. 3D). We observed that diphenylmethane compounds such as MBPE and tesmilifene (DPPE) induced similar differentiation effects as PBPE. SERMs including clomiphene, CI-628, 4OH-tamoxifen, and raloxifene are potent inducers of MCF-7 differentiation, whereas 17β-estradiol, which is not a ligand of the AEBS, has no effect. All these active compounds are amphiphilic cationic



D

	[drug] In μM	AEBS ligand affinity > 50 nM	ER affinity > 1μM	CAD	Lipid induction (x fold)	Specific cell cycle arrest	MFG induction > x 4
control					1.00		
PBPE	10	+		+	2.70	G1	+
DPPE	10	+		+	2.55	G1	+
MBPE	10	+		+	2.18	G1	+
Tam	2.5	+	+	+	1.96	G1	+
4-OH-tam	2.5	+	+	+	2.12	G1	+
Raloxifene	2.5	+	+	+	2.51	G1	+
CI-628	2.5	+	+	+	2.05	G1	+
Clomiphene	2.5	+	+	+	2.27	G1	+
Chloroquine	100			+	1.00		
17β-estradiol	0.1		+		1.00		
7-keto-cholesterol	5	+			2.05	G1	+
7-OH-cholesterol	5	+			1.86	G1	+
7-keto-cholestanol	5	+			2.24	G1	+
6-keto-cholestanol	5	+			2.01	G1	+
25-OH-cholesterol	10				1.00		

Figure 3. PBPE, tamoxifen, and AEBS ligands stimulate the expression and secretion of MFG. **A,** immunocytochemical analysis of the expression of MFG in MCF-7 cells treated with solvent vehicle, 10 μmol/L PBPE, and 2.5 μmol/L tamoxifen for 3 d. MFG was detected with a primary mouse polyclonal antibody and an anti-mouse fluorescein-labeled secondary antibody. The nucleus was stained with propidium iodide as described in Materials and Methods. Magnification, ×63 (for all images). **B,** PBPE and tamoxifen increase the expression of MFG. Effect of PBPE and tamoxifen on the expression of MFG was measured by Western blot analysis. Samples of NP-40 extracted cell lysates (100 μg protein) were loaded in each lane. MCF-7 cells were treated for 3 d with solvent vehicle, 10 μmol/L PBPE, or 2.5 μmol/L tamoxifen. **C,** analyses of secreted MFG were done by dot-blot analysis of aliquots of the culture medium from cells treated 1, 2, and 3 d with solvent vehicle, 10 μmol/L PBPE, or 2.5 μmol/L tamoxifen. Representative of at least three independent experiments. **D,** evaluation of the potency of AEBS ligands to induce differentiation characteristics in MCF-7 cells. MCF-7 cells were treated with the indicated concentrations for 48 h and analyzed. Compounds that display an affinity of >50 nmol/L for the AEBS are positive. Compounds that display an affinity of >1 μmol/L for the ER are positive. Amphiphilic cationic compounds (*ACD*) contain a cationic side chain grafted to a hydrophobic core. Quantitative analysis of lipid accumulation was done by extraction of the ORO-stained cells with isopropyl alcohol and measuring the absorbance at 492 nm as described in Materials and Methods.

drugs. Amphiphilic cationic drugs are known to induce lipidosis and the accumulation of intracytoplasmic vesicles (23). Therefore, we tested chloroquine, a prototypical amphiphilic cationic drug, with no affinity for the AEBS and showed that it did not induce differentiation characteristics in MCF-7 cells, showing that the amphiphilic cationic drug status is not related to the induction of differentiation in MCF-7 cells. The AEBS binds a subclass of oxysterols with high affinity (10) and we showed that these compounds are potent inducers of MCF-7 cell differentiation, whereas 25-hydroxycholesterol, which is an oxysterol that does not bind to the AEBS (10), did not induce differentiation characteristics in MCF-7 cells. The present data show that different structural classes of AEBS ligands induce differentiation characteristics in MCF-7 cells.

AEBS Ligands Induce Differentiation Characteristics in Different Breast Cancer Cell Lines

We have shown that PBPE and tamoxifen induced differentiation characteristics in MCF-7 cells that are ER-positive human breast cancer cells and observed the same effect in other ER-positive breast cancer cell lines such as ZR75-1 and TSA (data not shown). We next investigated whether PBPE and tamoxifen induced the same differentiation characteristics on two other more dedifferentiated breast cancer cell lines that expressed different a low level of ER (SKBr-3) or cells that are ER-negative (MDA-MB-468; ref. 24). The quantification of the AEBS is routinely done by saturation binding experiments using [^{3}H]tamoxifen on cell microsomal extracts (5). [^{3}H]tamoxifen binding variables were equivalent in MCF-7 and SKBr-3, whereas the number of AEBS was higher in the MDA-MB-468 and the affinity for [^{3}H]tamoxifen was weaker (Fig. 4A). Proliferation studies indicated that both SKBr-3 and MDA-MB-468 were less sensitive than MCF-7 cells to growth control induced by PBPE and tamoxifen (Fig. 4A). Cell cycle analysis showed that 3-day treatment with 20 μmol/L PBPE or 5 μmol/L tamoxifen induced an accumulation of cells in the G_0-G_1 (Fig. 4B). The treatment of cells with drugs increased the content of neutral lipids by a factor of 3.5 and 3.1 in MDA-MB-468 cells and by a factor of 2.6 and 2.5 in SKBr-3 cells, respectively, compared with controls (Fig. 4C). In addition, Western blot analyses showed that PBPE and tamoxifen stimulated the expression of MFG by a factor of 1.5 and 2.3 in MDA-MB-468 and by a factor 1.4 and 2.1 SKBr-3 cells, respectively (Fig. 4D). These data indicate that the stimulation of the expression of MFG in these cells by AEBS ligands at cytostatic concentrations is less pronounced than in MCF-7 cells. We observed that increasing the concentrations of PBPE and tamoxifen increased the number ORO-labeled vesicles and the percentage of cells in the G_0-G_1 phase of the cell cycle in MCF-7 cells (Supplementary Fig. S3A),[7] MDA-MB-468 cells (Supplementary Fig. S3B),[7] and SKBr-3 cells (Supplementary Fig. S3C).[7] Together, these data indicate that PBPE and tamoxifen are more potent to induce differentiation in breast cancer cell lines that expressed ER than in cell line that expressed low amount or no ER.

Sterols That Accumulated with AEBS Ligand Treatment Induce Differentiation and Growth Control of MCF-7 Cells

We then evaluated whether the main primary sterol metabolites accumulated after PBPE and tamoxifen interaction with the AEBS, zymostenol or DHC, induced differentiation characteristics and growth control in MCF-7 cells. These two sterols were purified by high-performance liquid chromatography (2) to eliminate putative toxic autoxidation products. Cell cycle analyses showed that treatment of the cells with 20 μmol/L zymostenol or DHC induced 67 ± 1% and 60 ± 2% accumulation of cells in the G_0-G_1 phase of the cell cycle, respectively, compared with 43 ± 1% for the control cells (Fig. 5A). This indicates that zymostenol and DHC induced a cell cycle arrest in the G_0-G_1 phase. In Fig. 5B, we show that the treatment of MCF-7 cells with 20 μmol/L zymostenol for 2 days increased the number of ORO-positive cells from 24 ± 6% in untreated cells to 75 ± 2%. DHC (20 μmol/L) induced a similar effect (Fig. 5B). Because these data indicated that these two metabolites increased the accumulation of neutral lipids, we determined the nature of the accumulated lipids. Zymostenol and DHC induced a 1.58- and 1.36-fold increase in the accumulation of free sterols, a 3.61- and 1.81-fold increase in the accumulation of esterified sterols, and a 3.7- and 2.3-fold increased accumulation of triacylglycerols, respectively, compared with untreated MCF-7 cells (Fig. 5C). In addition, both compounds increased the expression of MFG in MCF-7 cells compared with control cells (Fig. 5D). These data indicated that zymostenol and DHC induced similar differentiation characteristics in MCF-7 cells and growth control as those obtained with PBPE or tamoxifen.

Induction of the Differentiation Characteristics of MCF-7 Cells by AEBS Ligands, Zymostenol, and DHC Is Inhibited by Vitamin E

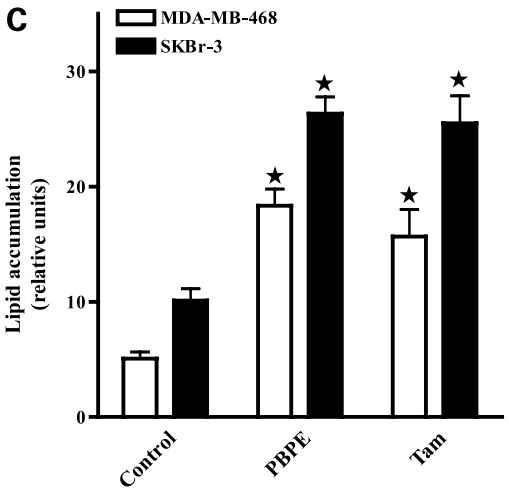
We reported previously that the accumulation of zymostenol consequent to PBPE or tamoxifen treatment of MCF-7 cells reached a maximum at 24 h and then decreased dramatically after 72 h of treatment. This decrease was correlated with the appearance of oxysterols and cell growth control (2). Based on these results, we evaluated here whether oxidation could be involved in the effects observed with PBPE and tamoxifen. As shown in Fig. 6A, 72 h treatment of MCF-7 cells with PBPE and tamoxifen induced a 51.1% and 43.5% diminution of the amount of zymostenol, respectively, compared with the amount accumulated at 24 h. The same experiment carried out in the presence of 500 μmol/L vitamin E completely reversed the diminution of the amount of zymostenol and confirmed its transformation by oxidation. We next tested the importance of oxidation on the growth control and the lipid accumulation induced by PBPE, tamoxifen, zymostenol, and DHC by determining the effects of these compounds in the presence of vitamin E. Treatment with vitamin E (500 μmol/L) totally inhibited the growth control (Fig. 6C) and the induction of triacylglycerol biosynthesis (Fig. 6B) by tamoxifen, PBPE, zymostenol, and DHC. These

A

	AEBS		EC_{50} (μM)	
	K_d (nM)	B_{max} (fmol/mg pr)	PBPE	Tam
MCF-7	5.3 ± 1.9	1534 ± 69	10.9 ± 4.2	2.5 ± 1.2
MDA-MB-468	9.4 ± 2.0	2604 ± 117	18.6 ± 3.1	6.8 ± 2.4
SKBr-3	6.8 ± 1.3	1627 ± 94	14.3 ± 4.8	5.4 ± 2.8

B

	Cell cycle								
	control			PBPE			Tam		
	G0-G1	S	G2+M	G0-G1	S	G2+M	G0-G1	S	G2+M
MCF-7	43 ± 1	48 ± 1	9 ± 2	76 ± 2	17 ±2	7 ± 2	73 ± 2	18 ± 1	9 ± 1
MDA-MB-468	46 ± 2	46 ± 1	8 ± 2	77 ± 3	15 ± 1	8 ± 2	78 ± 2	16 ± 2	6 ± 1
SKBr-3	48 ± 1	44± 2	9 ± 1	73 ± 7	18 ± 1	9 ± 1	72 ± 3	19 ± 1	9 ± 2



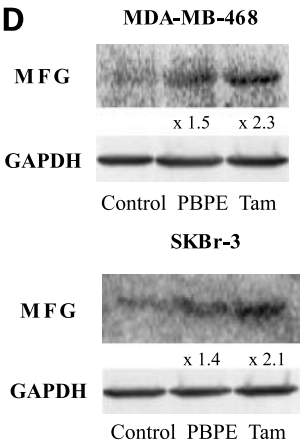


Figure 4. PBPE and tamoxifen induce differentiation characteristics through a mechanism that is independent of ER. **A,** AEBS density (B_{max}) values for MCF-7 cells were determined from saturation binding experiments with [^{3}H]tamoxifen. B_{max} values for MDA-MB-468 and SKBr-3 cells were estimated from the specific binding of 10 concentrations of [^{3}H]tamoxifen (0.1-1,000 nmol/L) under the conditions described in Materials and Methods. The sensitivity of cells for drugs were measured by treating them for 2 d with increasing concentrations of PBPE and tamoxifen from 0.5 to 50 μmol/L. Cells were harvested and counted on a Coulter counter. EC_{50} was measured as described in Materials and Methods. **B,** MCF-7 cells were treated with 10 μmol/L PBPE and 2.5 μmol/L tamoxifen for 3 d. MDA-MB-468 and SKBr-3 were treated with 20 μmol/L PBPE and 5 μmol/L tamoxifen for 3 d. Cell cycle distribution was measured as described in Materials and Methods by FACS flow analysis using the Becton Dickinson FACS system. **C,** lipid accumulation in MDA-MB-468 and SKBr-3 cells treated for 3 d with 20 μmol/L PBPE or 5 μmol/L tamoxifen. Mean ± SE obtained with the three radioligand concentrations. Quantitative analysis of lipid accumulation was done by extracting the ORO-stained cells with isopropyl alcohol and measuring the absorbance at 492 nm as described in Materials and Methods. **D,** MFG expression in MDA-MB-468 and SKBr-3 cells treated for 3 d with 20 μmol/L PBPE or 5 μmol/L tamoxifen was done as described in the legend of Fig. 3. ★, $P < 0.001$, compared with control; ★★, $P < 0.0001$, compared with control.

data underline the importance of the oxidative processes during the induction of MCF-7 cell differentiation and growth control by the AEBS ligands and by the primary sterol metabolites that are accumulated.

Discussion

The objective of our work was to define the nature of the growth control of breast cancer cells mediated by AEBS ligands and to determine if the metabolism of cholesterol was involved in this effect. We show that PBPE, a selective AEBS ligand (devoid of SERM activities), exerts differentiating activities by arresting breast cancer cells in the G_0-G_1 phase of the cell cycle, by inducing morphologic changes, and the accumulation and secretion of lipids and milk fat globule proteins that are characteristics of differentiated and lactating mammary epithelial cells. We report that tamoxifen induced the same differentiation characteristics as PBPE, confirming that the AEBS mediates part of the effects of tamoxifen. The induction of this process was observed with different structural classes of high-affinity AEBS ligands including drugs of clinical value such as raloxifene and tesmilifene as well as certain natural substances such as 7-ketocholesterol, showing that these effects can be generalized to high-affinity binding to the AEBS. The AEBS ligands PBPE and tamoxifen induced differentiation characteristics in ER-positive cell lines and ER-negative cell lines. ER-negative cells were, however, less sensitive than ER-positive cell lines, suggesting that drugs are more active on the less dedifferentiated cell lines. We showed that ER-negative MDA-MB-468 cells expressed 1.7-fold the amount of AEBS found in MCF-7 cells with a weaker affinity, which might reflect a difference in the expression of D8D7I and DHCR-7, the subunits of the AEBS. The AEBS found in SKBr-3 are similar to those found in MCF-7 cells, but these cells show a constitutive production of lipids, suggesting a difference in lipogenesis pathways compared with MCF-7.

Zymostenol and DHC are the substrates of D8D7I and DHCR-7, which are the two subunits of the AEBS. Zymostenol and DHC accumulate in MCF-7 cells treated with AEBS ligands (2). Importantly, we showed that the induction of cell differentiation characteristics and cell growth control are induced by zymostenol and DHC. This supports a mechanism by which AEBS ligands induced breast cancer cell differentiation through the intracellular

production of cholesterol precursors. We showed in a previous article that zymostenol first accumulated in MCF-7 under PBPE or tamoxifen treatment and then disappeared progressively after 48 h in favor of oxysterols (2). In the present study, we show that the antioxidant vitamin E inhibits the oxidative metabolism of zymostenol and its capacity to stimulate the appearance of differentiation characteristics in MCF-7. We further established that vitamin E inhibits the induction of the differentiation of MCF-7 cells by PBPE, tamoxifen, and DHC. These results indicate that sterol autoxidation products are involved in the pharmacologic effects mediated by the AEBS; thus, the activity of AEBS ligands is dependent on sterol oxidation. Autoxidation of sterols is produced by reactive oxygen species. Tamoxifen and 7-ketocholesterol have been shown to stimulate the production of reactive oxygen species through the stimulation of the expression NADPH oxidase in human hepatoma cell lines (25) and in mouse macrophages (26). NADPH oxidase has been reported to be inducible in MCF-7 cells (27), which makes its stimulation by AEBS ligands plausible in human breast cancer cells. Importantly, our work has established that the presence of the AEBS and the accumulation of sterol precursors are necessary, but not sufficient, to trigger the differentiation and the growth control of breast cancer cells induced by AEBS ligands and required a reactive oxygen species-producing system to transform sterol metabolites into oxysterols.

The induction of triacylglycerol by AEBS ligands is consistent with the production of oxysterols because they are known to control lipid metabolism through different mechanisms (11). They can bind liver X receptors and stimulate the expression of lipogenic enzymes and ATP-binding cassettes transporters A1 and G1 (ABCA1 and ABCG1) that are involved in the efflux of lipid, sterols, and oxysterols (28, 29). Liver X receptors have recently been shown to be involved in the normal physiologic processes of lactation (30). Moreover, the liver X receptor α subtype

A

Cell cycle								
control			Zymo			DHC		
G0-G1	S	G2+M	G0-G1	S	G2+M	G0-G1	S	G2+M
43 ± 1	48 ± 1	9 ± 2	67 ± 1	25 ±2	8 ± 2	60 ± 2	31 ± 1	9 ± 1

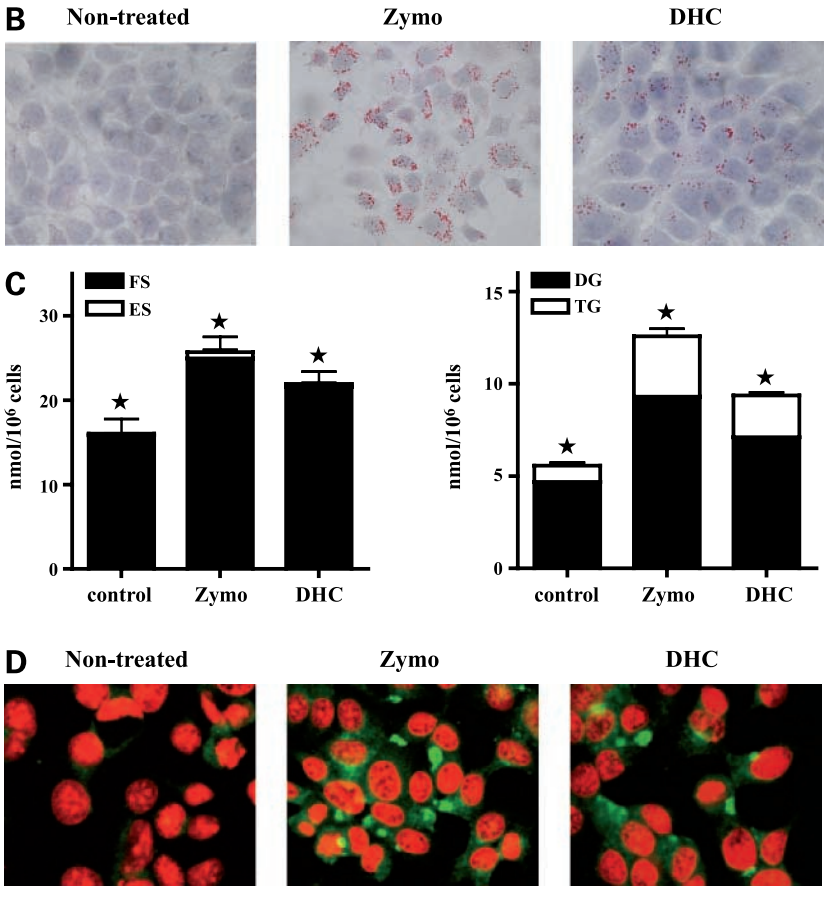


Figure 5. Intermediates of cholesterol biosynthesis that accumulate under AEBS ligand treatments induce differentiation characteristics in MCF-7 cells. **A,** zymostenol (*Zymo*) and DHC were incubated with MCF-7 cells at 20 μmol/L for 3 d. Cell cycle distribution was measured as described in Materials and Methods by FACS flow analysis using the Becton Dickinson FACS system. **B,** morphologic and biochemical changes were evaluated by light microscopy (×40) of ORO-stained cells counterstained with Mayer's hematoxylin as described in Materials and Methods. **C,** characterization and quantification of free sterols and esterified sterols and triacylglycerols present in MCF-7 cells were done as described in Materials and Methods. **D,** immunocytochemical analysis of the expression of MFG in MCF-7 cells treated with solvent vehicle, 20 μmol/L zymostenol, or DHC for 3 d. MFG was detected with a primary mouse polyclonal antibody and an anti-mouse fluorescein-labeled secondary antibody. The nucleus was stained with propidium iodide as described in Materials and Methods. Magnification, ×63 (for all images). ★, $P < 0.001$, compared with control.

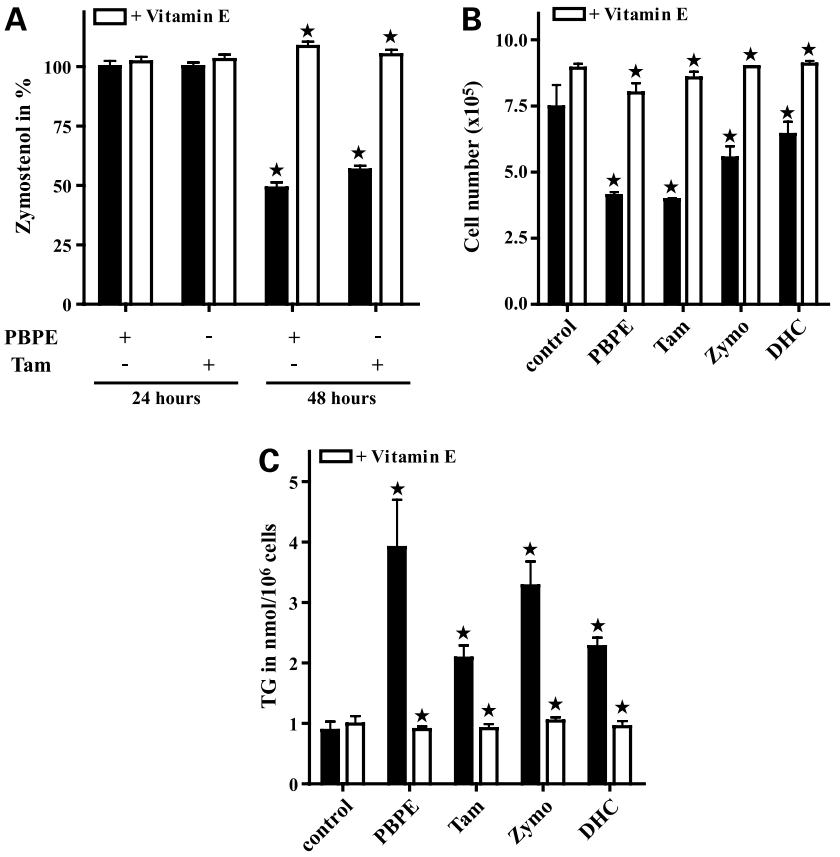


Figure 6. Vitamin E inhibits MCF-7 cell differentiation characteristics triggered by PBPE, tamoxifen, zymostenol, and DHC. **A,** MCF-7 cells were treated for 24 and 48 h with 10 μmol/L PBPE, or 2.5 μmol/L tamoxifen with or without 500 μmol/L vitamin E. Zymostenol was quantified as described in Materials and Methods. MCF-7 cells were treated for 3 d with 10 μmol/L PBPE, 2.5 μmol/L tamoxifen, 20 μmol/L zymostenol, or DHC in the presence or absence of 500 μmol/L vitamin E. **B,** cell numbers were estimated by counting with a Coulter counter. **C,** triacylglycerol content was measured as described in Fig. 2. Representative of at least three independent experiments. ★, $P < 0.001$, compared with control.

has been reported to be expressed in normal human breast, whereas its expression varied in breast cancer cell lines (31). We have observed that pharmacologic activators of liver X receptors such as T0901317 stimulate lipogenesis in MCF-7 cells (data not shown), suggesting that they might participate in the breast cell differentiation processes stimulated by AEBS ligands. Alternatively, oxysterols can bind to the oxysterol binding protein (OSBP)-related proteins (32). OSBP-related proteins constitute a family of cytosolic proteins involved in lipid metabolism (33). Interestingly, recent studies have shown that OSBPs can inhibit the catalytic activity of PP2A phosphatases that are involved in the control of the phosphorylation of Akt (34). Akt has been reported to control lipid metabolism during lactation in mice (35, 36), and okadaic acid, an inhibitor of PP2A, stimulates the differentiation of breast cancer cells (37). This suggests that oxysterols can control lactation processes in normal breast and in breast cancer cells through OSBP-related proteins by inhibiting the dephosphorylation of Akt. It remains to determine whether the liver X receptors or OSBP-related proteins pathways or both are involved in the induction of the differentiation of breast cancer cells by AEBS ligands. It remains to be studied what is the level of expression of OSBP-related proteins and proteins involved in lipid sterol efflux in these cells.

Tesmilifene (DPPE) is a structural analogue of PBPE that was brought up to a phase III clinical trial in association with cytotoxic drugs for breast cancer treatment (38). The rationale for this combination was the observation that tesmilifene sensitized tumor cells to chemotherapy by reversing their multidrug-resistant phenotype (39). Anticancer drugs are known to induce reactive oxygen species production in tumor cells (40), and we have observed that the combination of AEBS ligands with agents that stimulate the production of reactive oxygen species such as proinflammatory cytokines and cytotoxic drugs potentiate the growth control and the active cell death induced by AEBS ligands underlying the existence of an additional mechanism involved in this combination therapy.[8]

Breast differentiation therapy is a recent paradigm that emerged from epidemiologic studies showing that full-term pregnancy and breast-feeding protects the breast against neoplastic transformation (41). This leads to the concept that drugs inducing differentiation of breast tumors can be used as chemopreventive agents (42). The mechanism we describe here supports the possibility that modulation of cholesterol metabolism in tumor cells through the binding to the AEBS can be involved in the chemopreventive action of SERMs in addition to

[8] de Medina et al., unpublished results.

ER. The chemopreventive properties of tamoxifen and raloxifene have recently been confirmed despite the risk of endometrial carcinoma that is related to their SERM activity (43, 44). These data raise new insights into the mechanism that makes SERMs effective in decreasing the risk of breast cancer. Moreover, the development of selective AEBS ligands as curative and chemopreventive agents with no risk of endometrial cancer should constitute a helpful new strategy for breast cancer treatment and will require further investigations.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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