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Research Article

2-Methoxyestradiol Inhibits the Anaphase-Promoting Complex and Protein Translation in Human Breast Cancer Cells

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Abstract

2-Methoxyestradiol (2ME2), an estradiol metabolite with antiproliferative and antiangiogenic activities, is in phase I/II clinical trials for breast cancer. 2ME2 inhibits microtubule polymerization and causes cells to arrest in G_2-M. The purpose of this study was to further elucidate the molecular mechanism of 2ME2. MDA-MB-435 breast cancer cells were treated with 2ME2 (2 μmol/L) or vehicle alone. RNA was extracted and genomic profiling was done using 22k Agilent microarrays. Expression Analysis Systematic Explorer was used to determine enrichment of Gene Ontology categories. Protein isolates were subjected to Western blot analysis. Protein synthesis was measured with a [^{35}S]methionine pulse assay. An MDA-MB-435 cell line with two β-tubulin mutations (2ME2R) was used to determine whether novel mechanisms were tubulin-dependent. Gene Ontology categories enriched include genes that regulate the mitotic spindle assembly checkpoint, apoptosis, and the cytosolic ribosome. The target of the mitotic spindle assembly checkpoint is the anaphase-promoting complex (APC). APC inhibition was confirmed by measuring protein levels of its targets securin and cyclin B1, which were increased in 2ME2-treated cells. Because gene expression in the cytosolic ribosome category was decreased, we evaluated whether 2ME2 decreases protein translation. This was confirmed with a pulse assay, which showed decreased isotope incorporation in 2ME2-treated cells, which was maintained in the tubulin-resistant 2ME2R cells. APC inhibition was not maintained in 2ME2R cells. 2ME2 induces tubulin-dependent cell cycle arrest through regulation of genes involved in the mitotic spindle assembly checkpoint, which results in inhibition of the APC and tubulin-independent inhibition of protein translation. [Cancer Res 2007;67(2):702–8]

Introduction

2-Methoxyestradiol (2ME2) is orally active and has both antitumor and antiangiogenic activities *in vivo* (1, 2) and is currently in phase I/II clinical trials for cancer (3, 4). Although an estrogen metabolite, 2ME2 is not an estrogen receptor α or β agonist, and its antiproliferative and apoptotic activities (5–7) are independent of the interaction with those receptors (8). The antiangiogenic activity of 2ME2 is, at least in part, mediated through down-regulation of hypoxia-inducible factor-1α, which inhibits hypoxia-inducible factor-1–induced transcriptional activation of vascular endothelial growth factor expression (9). Inhibition of hypoxia-inducible factor-1 takes place downstream of the 2ME2/tubulin interaction, as disruption of interphase microtubules is necessary for hypoxia-inducible factor-1α down-regulation (9).

2ME2 causes cell cycle arrest at G_2-M (10–12), and it has been proposed that cell cycle arrest is mediated by the ability of 2ME2 to bind to the colchicine binding site of tubulin with impairment of spindle formation (11, 13); however, this finding has been disputed (10) and the process by which 2ME2 inhibits tumor growth is still not well understood.

Here we report further insight into the mechanism of cell cycle regulation by 2ME2 through inhibition of the anaphase-promoting complex (APC), a ubiquitin ligase of which the activation is required for separation of sister chromatids, which are the the target of the mitotic spindle assembly checkpoint. We also describe the identification of a novel mechanism of 2ME2-induced inhibition of protein translation via a tubulin-independent pathway.

Materials and Methods

Cell culture and proliferation assays. MDA-MB-435 human breast cancer cells were cultured in DMEM with 10% fetal bovine serum (Life Technologies, Inc., Carlsbad, CA) with 100 units of penicillin + streptomycin (Life Technologies). 2ME2R, a stable 2ME2-resistant MDA-MB-435 human cancer cell line with two acquired tubulin mutations (14), and the control parental MDA-MD-435 (P435) were characterized as previously described (14). 2ME2R and P435 cells were cultured in DMEM with 1% HEPES with 10% fetal bovine serum, 1 mmol/L sodium pyruvate, 1 mmol/L MEM nonessential amino acids and vitamins, and 1% penicillin/streptomycin. 2ME2R cells were maintained in 2ME2 (2 μmol/L).

To determine the IC_{50} value for 2ME2, cell proliferation assays were done in the following manner: MDA-MB-435, P435, and 2ME2R cells (20,000 per well) were plated in a 24-well plate and incubated for 24 h in the absence of 2ME2. 2ME2 [Sigma (St. Louis, MO) or EntreMed, Inc. (Rockville, MD)] was suspended in DMSO and serially diluted in media to make final concentrations ranging from 0.05 to 200 μmol/L. The final DMSO concentration was <1% by volume. The drug mixtures or solvent alone was added to each well 24 h after being seeded. Control wells for 2ME2 contained 1% DMSO by volume. After 48 h, cells were trypsinized and counted using a hemocytometer. The readings obtained for each concentration tested are from averages of three wells. Each experiment is expressed as a percentage of control, and the results are averages of at least three experiments. IC_{50} values were determined from dose-response curves using GraphPad Prism (San Diego, CA).

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Conflict of interest: Dr. George Sledge is a paid consultant for EntreMed, Inc.

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Cell culture and RNA extraction. MDA-MB-435 was plated (2.5×10^6 cells) in 75-cm^2 flasks or 100-mm plates in DMEM with 10% fetal bovine serum and 100 units of penicillin-streptomycin (Life Technologies). After 24 h, the medium was changed and cells were cultured with 2ME2 (2 μmol/L) or 1% DMSO by volume. After 48 h, total RNA was extracted using the Qiagen RNeasy Kit and purified with QIAquick PCR Purification Kit (Qiagen, Germantown, MD). The integrity and relative contamination of mRNA with rRNA were tested using the RNA 6000 Nano LabChip (Agilent Technologies, Santa Clara, CA) and Agilent 2100 bioanalyzer (Agilent Technologies). Technical replicates were done four times each to give a significant level of confidence for the average experimental-to-control intensity ratio for each gene.

cDNA synthesis and microarray hybridization. RNA from cells was reverse transcribed into cDNA labeled with Cy5-dUTP (Amersham Biosciences, Buckinghamshire, England) and into Cy3-labeled common reference sample that is a modified version of the Stratagene Human Universal (15) as previously described (16). Microarray hybridizations were done using 22k Agilent Human oligonucleotide microarrays. After hybridization, the arrays were scanned with Axon Gene Pix 4000 scanner (Axon Instruments, Inc., Foster City, CA). The images were analyzed using Gene Pix Pro 6.0 software (Axon Instruments).

Data normalization, preprocessing, and statistics. Gene expression values were quantified by the log 2 ratio of red channel intensity versus green channel intensity, followed by LOWESS normalization to remove the intensity-dependent dye bias and variation (17). UNC Microarray database[8] was used for filtering and preprocessing.[9] A two-class Significance Analysis of Microarrays (SAM[10]; refs. 18, 19) was done to identify significantly differentially expressed genes between arrays from treated and control cells.

To interpret the gene lists derived from the results of SAM and to convert the gene list into biological themes, we applied the Expression Analysis Systematic Explorer (EASE)[11] analysis. If genes in a biological category are significantly enriched in the SAM-derived gene list, then that biological category may be involved in the biological system. EASE can assign a score to quantify how much confidence we have about the discovery.

Cell cycle analysis by flow cytometry. Cell cycle analysis was done as previously described (20). Briefly, cells (2×10^5) were plated on a 60-mm plate, harvested by trypsinization, pelleted, and resuspended in 1 mL of PBS. After spinning, RNase A was added (final concentration, 2 mg/mL). Cells were further stained with 50 μg/mL propidium iodide solution. Cell cycle analysis was carried out with a Becton Dickinson (Bedford, MA) FACScan flow cytometer. Data were analyzed with the Modfit LT software (Verity Software House, Inc., Topsham, ME).

Apoptosis. The carboxyfluorescein FLICA apoptosis detection kit (Immunohistochemistry Technologies, LLC, Bloomington, MN) was used to measure typical apoptosis, atypical apoptosis, and necrosis (21). Briefly, 2×10^5 cells were grown overnight on a 60-mm plate and treated with 2ME2 (1 and 5 μmol/L) for 24 or 48 h. Both attached and floating cells were collected by trypsinization and incubated with carboxyfluorescein-labeled pan-caspase inhibitor FAM-VAD-fmk for 2 h at 37°C. Labeled cells were rinsed twice in PBS and resuspended in 300 μL of PBS containing 0.3 μg of propidium iodide. Apoptotic cells were identified by FACScan analysis as previously described (21). All apoptosis assays were done thrice and representative data are presented.

Cell-surface staining for cyclin B1. Surface staining for cyclin B1 was done according to the manufacturer's instructions (fluorescein-FITC-conjugated cyclin B1 antibody reagent set, BD PharMingen, San Diego, CA). Briefly, cells were washed with PBS, adjusted to 10^6 cells per sample, and incubated with either FITC-conjugated mouse immunoglobulin G (IgG) as a control or cyclin B1 for 30 min at room temperature. Analysis was done by using the CellQuest software.

Western blotting. P435 or 2ME2R cells were seeded in T75 culture flasks in the absence of 2ME2. After 24 h, the medium was then replaced with a new medium containing either vehicle (0.1% DMSO) or 5 μmol/L 2ME2 for 16, 24, or 48 h at 37°C. Whole-cell lysates were prepared by suspending the cells in lysis buffer (8 mol/L urea, 4% CHAPS, 15 mmol/L Tris-HCl) containing protease inhibitor cocktail (Roche, Nutley, NJ) and then briefly sonicated. Proteins (40 μg/lane) were resolved by 4% to 12% SDS-PAGE and electrotransferred to a polyvinylidene difluoride membrane. The membrane was then incubated with the securin primary rabbit antibody (4 μg/mL; Zymed Laboratories, San Francisco, CA), followed by securin secondary goat anti-rabbit horseradish peroxidase (HRP)–conjugated antibody (1:10,000; Chemicon International, Temecula, CA) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primary mouse antibody (1:5,000; Chemicon International), and then by GAPDH secondary sheep anti-mouse HRP-conjugated antibody (1:10,000; Amersham Biosciences), and finally electrochemiluminescence detection was done. Proteins were normalized on GAPDH expression in the same lane. Relative absorbances (arbitrary units) were normalized against those of each control group. Results are expressed as the mean ± SE. Statistical analysis was done using a one-tailed unpaired t test, and $P < 0.05$ was considered significant.

Antibodies against cyclin B1 and cdc2 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies against phospho-cdc2 (Thr^{161} and Tyr^{15}) and phospho-Bcl-2 were from Cell Signaling Technology (Danvers, MA). Phospho-Bcl-2 and total Bcl-2 were from BD PharMingen. All the primary antibodies were used at 1:1,000 dilution. Secondary antibodies against rabbit were used at 1:10,000 whereas secondary antibodies against mouse were used at 1:2,000 (Amersham, Piscataway, NJ).

APC ubiquitination assay. Ubiquitination assays were carried out as described (22). The substrate Clb2 (pRSETClb2), the coactivator Cdh1 (pET28-His_6-Cdh1), and the E2 Ubc4 (pET28-His_6-Ubc4) were prepared using the TNT T7 Quick coupled *in vitro* transcription/translation kit (Promega, Madison, WI). APC (YKA156) was purified from budding yeast (W303 background) arrested in late M phase and ~0.1 pmol was used in each reaction. For APC purification, cells from the strain YKA156 (*MATa bar1::URA3 cdc15-2 ura3 leu2 trp1 CDC27-3FLAG::KanMX4*) were arrested in late M phase during mid-log phase growth. Late M phase arrest was achieved by shifting YKA156 cultures (*cdc15-2* allele) from 25°C (permissive) to 37°C (restrictive). Cells were lysed in 1 volume of APC-C lysis buffer [25 mmol/L HEPES-NaOH (pH 7.5), 400 mmol/L NaCl, 10% glycerol, 0.1% Triton X-100, 0.1 mmol/L DTT, 0.5 mmol/L phenylmethylsulfonyl fluoride (PMSF), and complete protease inhibitor cocktail (Roche)] by vortexing for 45 min at 4°C in 1.7-mL microcentrifuge tubes containing 0.5 mL of 0.5-mm glass beads (23). Cell lysate was cleared by centrifugation at 16,000 × g for 15 min. Soluble extracts were pooled and cleared a second time at 5,000 rpm for 5 min. Extracts were incubated with EZView anti-FLAG M2 antibody-coupled resin (Sigma) for 1.5 h at 4°C, washed extensively with APC-C buffer, and eluted with FLAG peptide (250 μg/mL) in low-salt APC-C buffer [25 mmol/L HEPES-NaOH (pH 7.5), 150 mmol/L NaCl, 10% glycerol, 0.1% Triton X-100, 0.1 mmol/L DTT, z0.5 mmol/L PMSF]. Clb2 was radiolabeled with [^{35}S]methionine (Perkin-Elmer, Wellesley, CA). Stock solutions (20×) of 2ME2 were used and final concentrations in each reaction were as noted. Reactions were run on a 4% to 12% Bis-Tris NuPAGE gel (Invitrogen), dried, and imaged using Hyperfilm (Amersham Biosciences).

Protein translation assay. MDA-MB-435 and 2ME2R cells were seeded in T75 culture flasks. After 24 h, the medium was replaced with new medium containing either 2ME2 (2 μmol/L) or vehicle (DMSO) alone and was then treated for a period of 48 h. Cells were suspended in pulse media containing DMEM/MG132 (20 μmol/L)/[^{35}S]methionine (250 μCi/100 μL) with either 2ME2 or vehicle alone. The cell suspensions were incubated in a water bath at 37°C for periods of 5, 10, 15, and 30 min. Immediately after briefly vortexing, equal aliquots were then taken at each time point. Isotope incorporation was then stopped by placing each aliquot in 10× volume of ice-cold PBS containing 1 mg/mL methionine. Cells were pelleted and resuspended in lysis buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100) containing proteinase inhibitor cocktail (Roche). Cells were then lysed on ice for 30 min, with vortexing every 10 min. The lysates were then spun at 16,000 × g for 15 min to pellet the insoluble fraction. Equal volumes of the soluble fraction were resolved

[8] http://genome.unc.edu/.
[9] Data associated with this study are available at http://genome.unc.edu/pubsup/breastTumor/.
[10] http://www-stat.stanford.edu/~tibs/SAM/.
[11] http://david.niaid.nih.gov/david/ease.htm.

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using 4% to 12% SDS-PAGE. Gels were then fixed, dried, and quantitated with PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Results

Proliferation assays. 2ME2 sensitivity in MDA-MB-435, 2ME2R, and P435 cells was determined by a 48-hour proliferation assay. IC_{50} values for 2ME2 were 1.3 μmol/L for MDA-MB-435, 99.5 μmol/L for 2ME2R cells, and 4.7 μmol/L for P435. This represents a 21-fold increase in resistance of 2ME2R cells to 2ME2 compared with P435 cells, which is consistent with the recently published report that characterized this new resistant cell line (14).

Identification of treatment response genes using cDNA microarrays. Gene expression was studied in MDA-MB-435 human breast cancer cells with and without the addition of 2ME2 (2 μmol/L) using human 22k Agilent microarrays. Using SAM, there was differential expression in 264 genes, of which 135 were up-regulated and 129 were down-regulated (Supplementary data). When categorized by function with EASE, there were changes in expression of genes involved in six Gene Ontology categories (Fig. 1*A*). We further investigated three of the Gene Ontology categories, cell cycle (Fig. 1*B, i*), apoptosis (Fig. 1*B, ii*), and cytosolic ribosome (Fig. 1*B, iii*), and evaluated whether their mechanisms were tubulin dependent.

Regulation of cell cycle and apoptosis by 2ME2 is tubulin-dependent. To determine whether regulation of cell cycle and apoptosis by 2ME2 is tubulin dependent, we first studied the cell cycle distribution in parental (P435) and 2ME2-resistant (2ME2R) cells by treating both cells with either 1 or 5 μmol/L 2ME2 and did a time course study. As shown in Fig. 2*A* and *B*, treatment of P435 with 2ME2 led to an accumulation of cells at the G_2-M phase of the cell cycle as early as 16 h (63.0% and 78.6% at 1 and 5 μmol/L, respectively). Whereas G_2-M arrest was reversible with 1 μmol/L at 24 and 48 h, the cells remained at G_2-M longer with 5 μmol/L treatment. In contrast, no major G_2-M arrest was observed in 2ME2R cells even after 48 h of 2ME2 treatment, indicating that 2ME2R cells are insensitive to 2ME2-induced G_2-M cell cycle arrest (Fig. 2*A* and *B*). The cell cycle profile for each cell line was also done at each time point in the absence of drug treatment. The profile was similar to time 0 (data not shown).

We further evaluated the sensitivity of these cells to apoptosis, which usually follows G_2-M arrest. We did the carboxyfluorescein FLICA assay to measure the level of active caspases and loss of plasma membrane integrity and to quantitate live, apoptotic, atypical apoptotic (apoptosis with loss of plasma membrane integrity), and necrotic cells (21). P435-sensitive cells exhibited increased typical and atypical apoptosis in a time-dependent manner in response to 2ME2 (Fig. 3). In contrast, 2ME2R cells failed to undergo apoptosis when treated with 2ME2.

Inhibition of the APC. Genes involved in the regulation of the mitotic spindle assembly checkpoint, *MAD2* (mitotic arrest deficient), *BUB1*(budding uninhibited by benzimidazole), and *CDC20*, were differentially expressed by microarray analyses in the 2ME2-treated

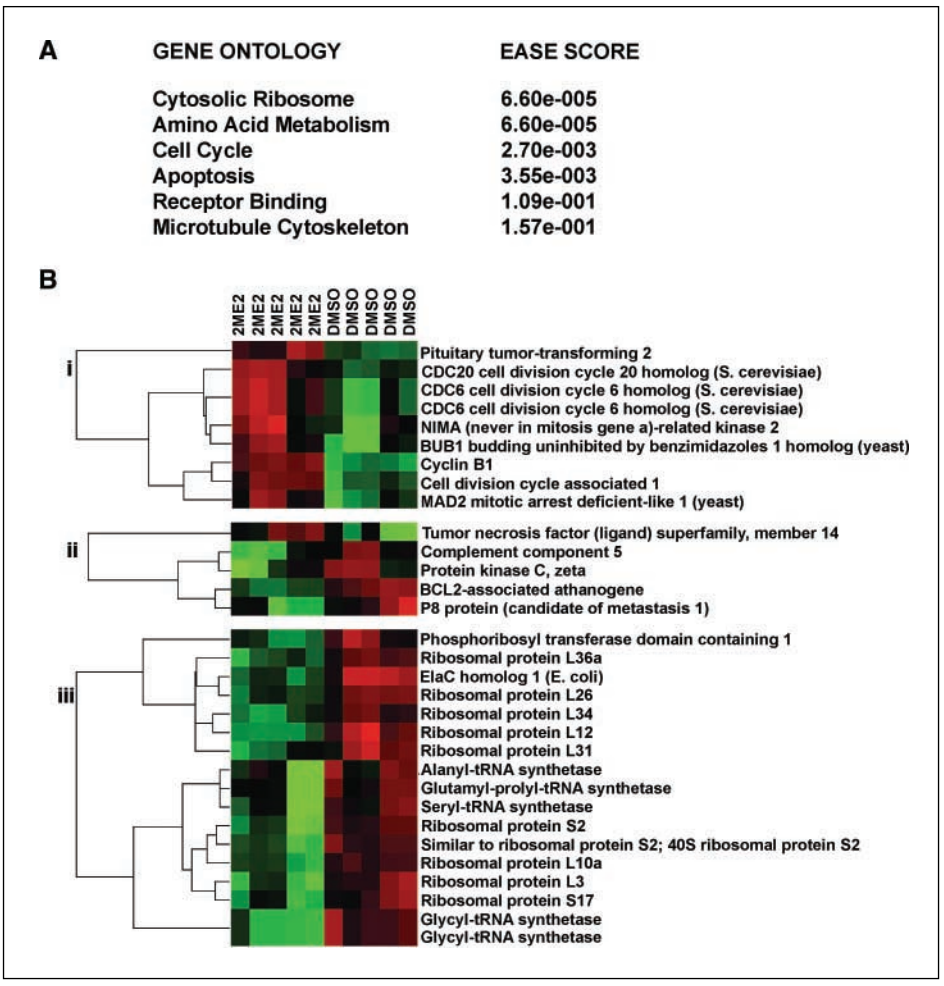

Figure 1. Gene Ontology categories differentially expressed in 2ME2-treated MDA-MB-435 cells. *A,* Gene Ontology categories differentially expressed in 2ME2-treated cells. *B, i,* expression of genes involved in the mitotic spindle assembly checkpoint in the regulation of cell cycle is increased. *ii,* expression of genes involved in apoptosis. *iii,* expression of genes involved in the cytosolic ribosome is decreased in 2ME2-treated cells. The complete data set is available online through the Gene Expression Omnibus (Series Record GSE5665, http://www.ncbi.nlm.nih.gov/geo) and through the University of North Carolina Microarray Database (http://genome.unc.edu).

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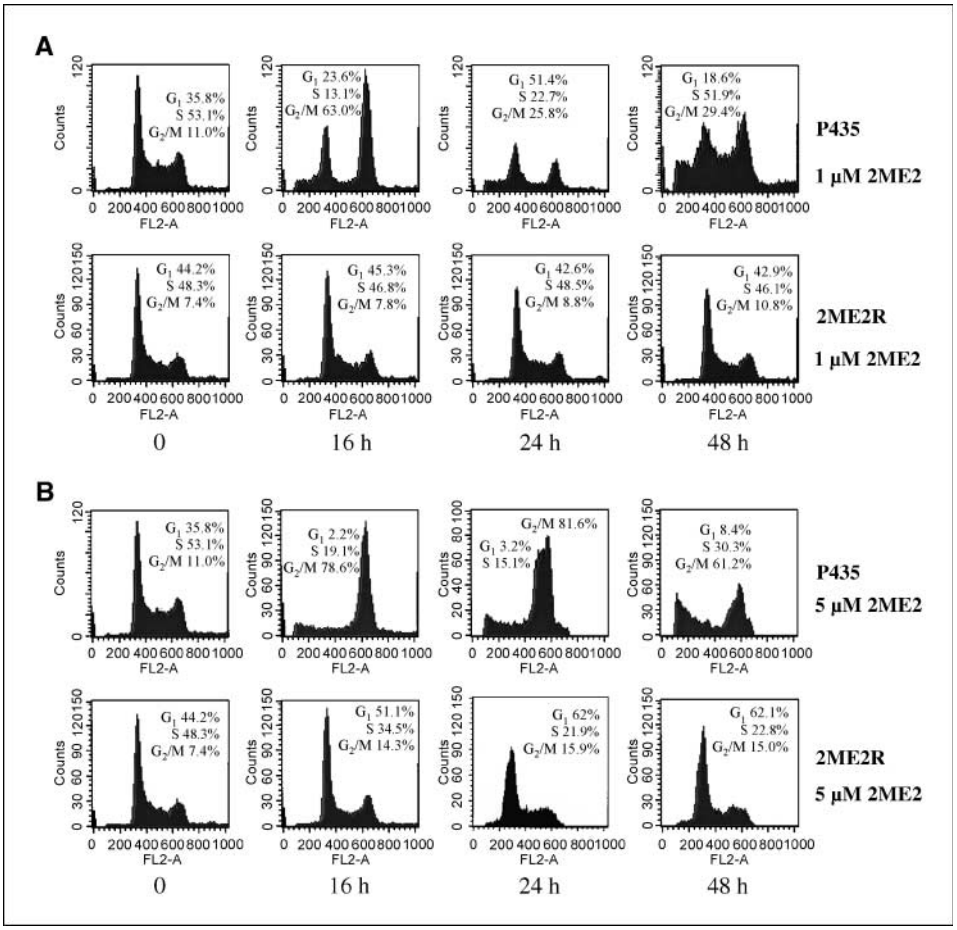

Figure 2. Cell cycle distribution of the 2ME2-sensitive parental P435 cells and 2ME2-resistant 2ME2R cells. Cells were treated with either 1 μmol/L (*A*) or 5 μmol/L (*B*) 2ME2 for the indicated time points, then trypsinized, fixed, and stained with propidium iodide. Cells at G_1, S, and G_2-M phases for each condition are shown in percentage.

MDA-MB-435 cells (Fig. 1*B, i*). The mitotic assembly checkpoint is controlled by the APC (24). To confirm the involvement of the genes that regulate the mitotic spindle assembly checkpoint, we analyzed the expression levels of securin and cyclin B1, the essential targets of the APC. Using Western blot analysis, we found statistically significant increases in protein levels of securin at 16 and 24 h (Fig. 4*A* and *C*) in 2ME2-treated P435 cells, providing evidence that the APC was inhibited. In contrast, securin was increased at 16 h in 2ME2-treated 2ME2R cells, but the increase was attenuated at 24 h and was significantly lower than the increase seen at 24 h in the P435 cells (Fig. 4*B* and *D*).

Next, we evaluated cyclin B1 in cells treated with 2ME2 (5 μmol/L). P435 cells and 2ME2R cells were analyzed by flow cytometry to determine the cyclin B1 expression throughout the cell cycle progression using FITC-labeled cyclin B1 antibody. Cyclin B1 expression at G_2-M phase was increased at 16 and 24 h in P435

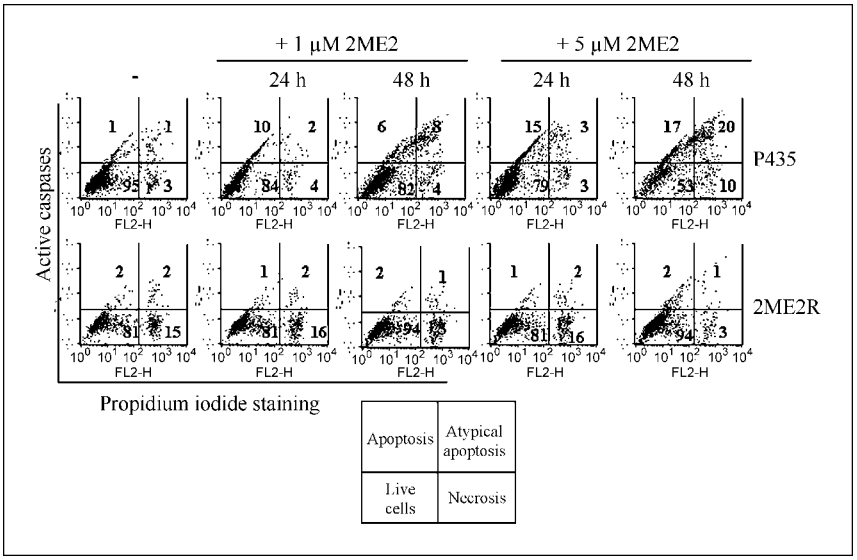

Figure 3. Apoptosis measured using the carboxyfluorescein FLICA assay kit (Immunohistochemistry Technologies). Briefly, 2×10^5 cells were plated overnight on a 60-mm plate and exposed to 2ME2 for 24 and 48 h. Using FACScan analysis, cells that are live and undergoing atypical or classic apoptosis or necrosis are shown in percentage.

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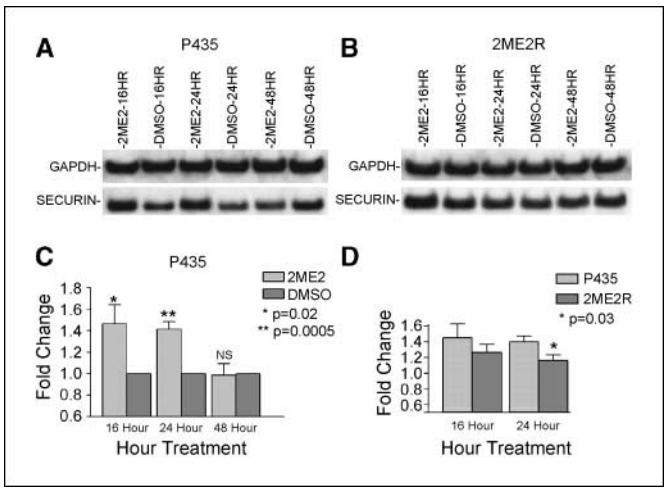

Figure 4. Securin protein expression is increased in 2ME2-treated breast cancer cells. P435 and 2ME2R cells were cultured with 2ME2 (5 μmol/L) or DMSO for 16, 24, or 48 h. Western blotting showed increased protein levels of securin in 2ME2-treated cells at 16 and 24 h compared with control in P435 (*A* and *C*), which is attenuated in 2ME2R (*B* and *D*) cells. Experiments were done in triplicate for all time points. *Bars*, SD.

cells, being 68.7% and 64.3%, respectively (Fig. 5*A*), but not in 2ME2R cells (Fig. 5*B*).

Similar results were confirmed by Western blotting of cyclin B1 (Fig. 5*C*). Consistent with flow cytometry data, protein levels of cyclin B1 were increased at both 16 and 24 h in P435 cells, whereas the increase was not obvious with 2ME2R cells. Cyclin B1 is associated with cdc2 protein. Comparison of phosphorylation status of cdc2, a G_2-M regulatory protein that controls the entrance to and exit from M-phase, showed that phosphorylation of Thr^{161} representing the active form was increased in response to 2ME2 treatment at 16, 24, and 48 h (which was not seen in 2ME2R cells), whereas phosphorylation of Tyr^{15} (inactive form) was decreased at the same time points. Furthermore, we observed the phosphorylation of Bcl-2 protein specifically in P435 cells when treated with 2ME2 at 16 and 24 h, consistent with an earlier report (25). Taken together, these results provide further support that regulation of cell cycle and apoptosis by 2ME2 is tubulin dependent.

2ME2 does not directly bind to the APC. To elucidate whether 2ME2 is *directly* binding to and inhibiting the APC, we evaluated the effect of 2ME2 on the APC in an APC ubiquitination assay. In this assay, the APC is isolated from yeast and incubated with coactivator Cdh1 and substrate Clb2, and, as a result, Clb2 is ubiquitinated. We found that the addition of 2ME2 did not inhibit ubiquitination of the substrate Clb2 (Supplementary data), which shows that 2ME2 does not directly bind to and inhibit the APC.

2ME2 inhibits protein translation. We next evaluated the effect of 2ME2 on protein translation because gene expression in the cytosolic ribosome category was decreased in response to 2ME2. We measured protein translation in MDA-MB-435 cells after incubation with 2ME2 (2 μmol/L) for 48 h, using a pulse assay measuring [^{35}S]methionine incorporation. We found a 25% decrease in the percentage increase of isotope incorporation in 2ME2-treated cells (P = 0.01; Fig. 6*A* and *B*). To determine whether

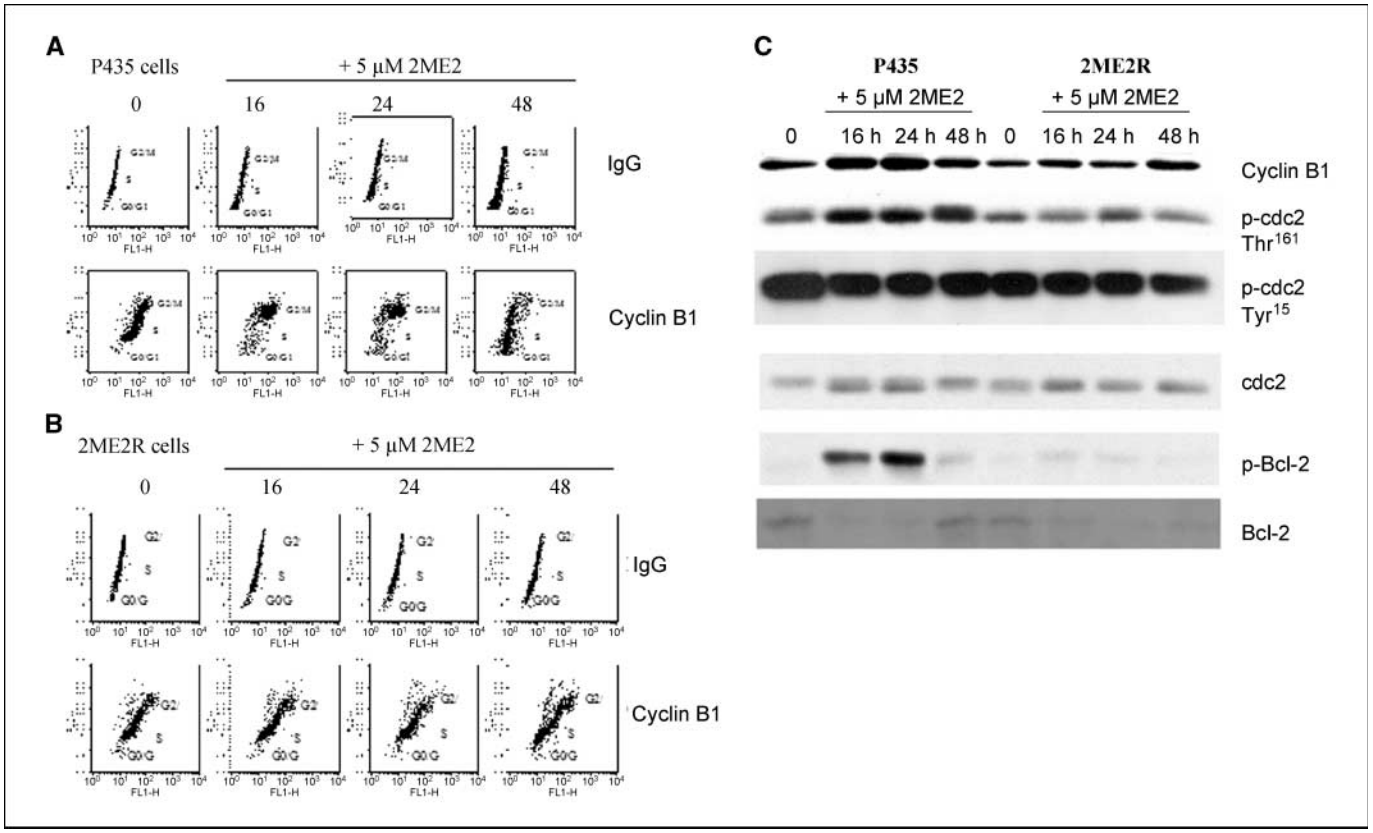

Figure 5. Flow cytometric evaluation of cyclin B1 expression. P435 (*A*) and 2ME2R (*B*) cells were analyzed to check the cyclin B1 expression throughout the cell cycle progression using a FITC-labeled cyclin B1 antibody. *C,* Western blot analysis was done with antibodies against cyclin B1, phospho-cdc2 (Thr^{161} or Tyr^{15}), and total cdc2, phospho-Bcl-2, and total Bcl-2.

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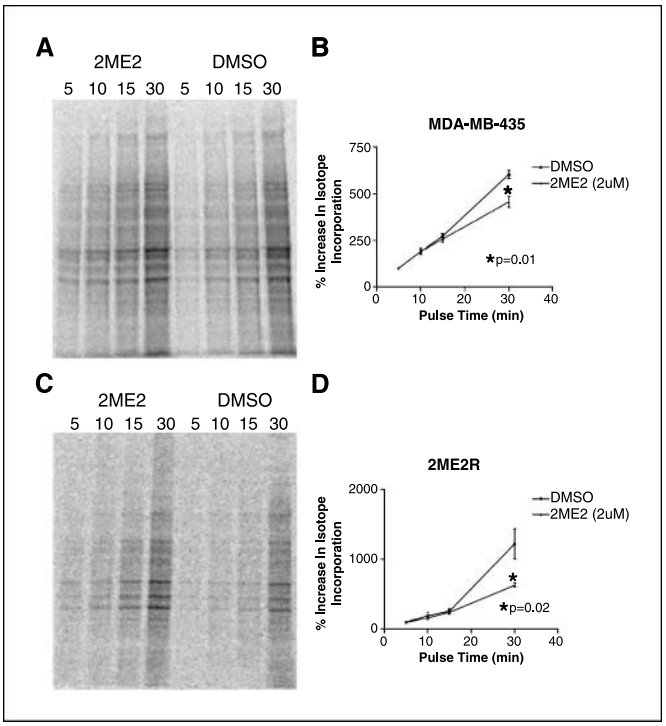

Figure 6. [^{35}S]Methionine pulse assay. Protein translation in MDA-MB-435 cells after incubation with 2ME2 (2 μmol/L) for 48 h was measured using a pulse assay using [^{35}S]methionine. There was a 25% decrease in the percentage increase of isotope incorporation in 2ME2-treated cells (P = 0.01; *A* and *B*) and a 52% decrease in the percentage increase of isotope incorporation in 2ME2-treated cells (P = 0.02; *C* and *D*), showing that 2ME2 inhibits protein translation in a tubulin-independent manner. *Bars*, SD.

inhibition of protein translation by 2ME2 was tubulin dependent, we next treated 2ME2R cells with 2ME2 (2 μmol/L) for 48 h and used a pulse assay. We found a 52% decrease in the percentage increase of isotope incorporation in 2ME2-treated cells (P = 0.02; Fig. 6*C* and *D*), showing that 2ME2 inhibits protein translation in a tubulin-independent manner.

Discussion

Several studies reported multiple functions of 2ME2, including disruption of microtubule dynamics by its binding ability to the colchicine binding site of tubulin, regulation of cell cycle and apoptosis, and dysregulation of hypoxia-inducible factor-1 (26). However, the mechanisms of actions of 2ME2 are still complex. D'Amato et al. (11) showed that 2ME2 binds to the colchicine binding site of tubulin, which suggested that mitotic arrest by 2ME2 was attributed to the inhibition of tubulin polymerization. However, Attalla et al. (10) subsequently showed that 2ME2 caused complete metaphase arrest at concentrations that do not inhibit the assembly of mitotic spindles; thus, whether or not the regulation of the cell cycle by 2ME2 is dependent on interaction with tubulin has remained unclear.

In the present study, we first sought to identify potential targets that were differentially regulated in response to 2ME2. Using long oligo-spotted microarrays, we found the cell cycle, apoptosis, and cytosolic ribosome Gene Ontology categories differentially expressed in 2ME2-treated MDA-MB-435 cells. Using a stable 2ME2-resistant cell line (2ME2R), we further explored whether these functions are tubulin dependent or not.

We have previously shown that this cell line derived from parental MDA-MB-435 (P435) has two acquired point mutations in the class I (M40) β-tubulin both at the DNA and protein levels (14). Immunofluorescence and *in vitro* polymerization assays showed that 2ME2-driven tubulin depolymerization is impaired in 2ME2R cells (14).

We studied 2ME2-mediated cell cycle events in this 2ME2R cell line and delineated tubulin-dependent versus tubulin-independent effects of 2ME2. We found that treatment of P435 with 2ME2 led to an accumulation of cells at G_2-M, but no major G_2-M arrest was seen in 2ME2R cells. We also found that P435 cells underwent apoptosis in a time-dependent manner in response to 2ME2, whereas 2ME2R cells did not undergo apoptosis when treated with 2ME2. Furthermore, we observed phosphorylation of Bcl-2 protein only in P435 cells, consistent with an earlier report (25). These data strongly suggest that regulation of cell cycle and apoptosis by 2ME2 is tubulin dependent.

Cell cycle–related genes that were increased in 2ME2-treated MDA-MB-435 cells included *MAD2, BUB1*, and *CDC20*, which are involved in the mitotic spindle assembly checkpoint. Cell cycle progression is monitored by the mitotic spindle assembly checkpoint, which monitors that all chromosomes have achieved bivalent attachment to microtubules (27). The target of the checkpoint control is the APC, a ubiquitin ligase of which the activation is required for separation of sister chromatids. Formation of MAD-BUB complexes is crucial for the checkpoint mechanism (27, 28). On activation of the mitotic spindle assembly checkpoint, MAD2 binds to and inhibits the APC, causing a metaphase arrest and preventing securin and cyclin B1 proteolysis by blocking its ubiquitination, which is critical in controlling the metaphase-anaphase transition (24, 27).

We found that securin and cyclin B1 proteins were increased in 2ME2-treated P435 cells, suggesting that the APC is inhibited. In contrast, we found that cyclin B1 was not increased in 2ME2R cells, and the increase in securin was attenuated in 2ME2R cells. Furthermore, using flow cytometry, we found that cyclin B1 levels were increased at G_2-M in 2ME2-treated P435 cells, but not in resistant 2ME2R cells. This suggests that 2ME2 inhibits APC in a tubulin-dependent manner.

Progression of prophase to metaphase is controlled by activation of the cdc2/cyclin B1 complex (29). Cdc2 (or $p34^{cdc2}$), a G_2-M regulatory protein that controls entrance to and exit from M-phase, is activated in P435 cells. Phosphorylation status of cdc2 showed that phosphorylation of Thr^{161} representing the active form was increased in response to 2ME2 treatment in P435 cells but not in 2ME2R cells, whereas phosphorylation of Tyr^{15} (inactive form) was decreased. This suggests that P435 cells had entered metaphase. However, further entry into the anaphase depends on the destruction of cyclin B1/cdc2 activity by APC. Based on the up-regulation of cyclin B1 and activation of cdc2, P435 cells were arrested at metaphase. Taken together, these results suggest that the presence of tubulin mutations in 2ME2R cells may affect the association of cyclin B1/cdc2 complex with the microtubules via inhibition of the APC, resulting in altered cell cycle response in 2ME2R.

To exclude the possibility that 2ME2 is *directly* binding to and inhibiting the APC, we evaluated the direct effect of 2ME2 on the APC in an APC ubiquitination assay. We found that the addition of 2ME2 in this assay did not inhibit ubiquitination of the substrate Clb2, which shows that 2ME2 does not directly bind to and inhibit the APC.

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Genes in the cytosolic ribosome category, which are responsible for the formation of the ribonucleoprotein complex, were significantly decreased in 2ME2-treated cells, and therefore we hypothesized that 2ME2 inhibits protein translation. In numerous cancers, general protein synthesis rates and the expression of several translation components are significantly elevated, supporting the potential importance of translational control in tumor progression (30). Using a [^{35}S]methionine pulse assay, we found an inhibition of protein translation in 2ME2-treated MDA-MB-435 cells. We also found that 2ME2 significantly inhibited protein translation in 2ME2R cells, showing that inhibition of protein translation by 2ME2 acts via a tubulin-independent pathway. Because 2ME2R cells are highly resistant to 2ME2, yet 2ME2 significantly inhibited protein translation, this suggests that the effects on protein translation are not important to the antimitotic effect of 2ME2.

In conclusion, our data suggest that the cell cycle, apoptotic, and APC regulatory effects of 2ME2 are tubulin dependent. These mechanisms are important aspects of the antimitotic effect of 2ME2, as they were diminished in a highly 2ME2-resistant cell line. We also discovered a novel mechanism of action of 2ME2: inhibition of protein translation by decreasing expression of genes involved in the formation of the ribonucleic protein complex via a tubulin-independent mechanism. Protein translation inhibition was maintained in a highly 2ME2-resistant cell line, and therefore this mechanism does not contribute to the antiproliferative effect of 2ME2. Whether this contributes to antitumor activity *in vivo* requires further investigation.

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