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## 2-Methoxyestradiol Up-Regulates Death Receptor 5 and Induces Apoptosis through Activation of the Extrinsic Pathway

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### ABSTRACT

2-Methoxyestradiol (2ME<sub>2</sub>), a natural metabolite of estradiol, is a potent antitumor and antiangiogenic agent. *In vitro*, 2ME<sub>2</sub> inhibits the proliferation of a wide variety of cell lines and primary cultures, and in numerous models *in vivo*, it has been shown to be an effective inhibitor of tumor growth and angiogenesis. 2ME<sub>2</sub> is currently in several Phase I and Phase II clinical trials under the name Panzem. Although various molecular targets have been proposed for this compound, the mechanism by which 2ME<sub>2</sub> exerts its effects is still uncertain. This study shows that 2ME<sub>2</sub> uses the extrinsic pathway for induction of apoptosis. 2ME<sub>2</sub> treatment results in up-regulation of death receptor 5 (DR5) protein expression *in vitro* and *in vivo* and renders cells more sensitive to the cytotoxic activities of the DR5 ligand tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). 2ME<sub>2</sub>-induced apoptosis requires caspase activation and kinetic studies show the sequential activation of caspase-8, caspase-9, and caspase-3. Blockage of death receptor signaling by expression of dominant-negative Fas-associated death domain severely attenuates the ability of 2ME<sub>2</sub> to induce apoptosis. Because 2ME<sub>2</sub> administration has not manifested dose-limiting toxicity in the clinic, DR5 expression may serve as a surrogate marker for biological response.

### INTRODUCTION

2ME<sub>2</sub><sup>3</sup> is a physiological metabolite of estradiol that is formed *in vivo* by sequential hydroxylation and methylation at the 2-position. Once dismissed as an inert metabolic end product of estradiol, 2ME<sub>2</sub> is now recognized as a potent p.o. active antiangiogenic and antitumor agent, which has virtually no toxicity. Although 2ME<sub>2</sub> has been speculated to be a natural chemopreventative agent (1, 2), the physiological function of this molecule remains undefined. We have recently shown that the actions of 2ME<sub>2</sub> are independent of the ERs. 2ME<sub>2</sub> has low binding affinity for both ER $\alpha$  and ER $\beta$ , does not use an ER for its antiproliferative activities, and neither ER agonists nor antagonists influence the activity of 2ME<sub>2</sub> (3). The antiproliferative activities of 2ME<sub>2</sub> have been attributed to several mechanisms, including effects on tubulin polymerization and depolymerization (4–6), up-regulation of p53 (7, 8), and inhibition of superoxide dismutase enzymatic activity (9). Although some of these mechanisms are sufficient to explain the activity of 2ME<sub>2</sub> in certain cases, there is presently no evidence for a common mechanism of action operative in all cells sensitive to 2ME<sub>2</sub>.

Apoptosis is an intracellular suicide program that is executed by the activation of caspases, a family of cytoplasmic cysteine proteases. Two major pathways, the intrinsic and the extrinsic apoptotic path-

ways, induce the caspase signaling cascade. The intrinsic pathway involves the cell sensing stress that triggers mitochondria-dependent processes, resulting in cytochrome *c* release and activation of caspase-9 (10). The extrinsic pathway involves activation of DRs such as Fas, TNF receptor 1, DR4, and DR5. Interaction with their respective ligands leads to a signal transduction cascade initiated by the recruitment of DR-associated molecules such as FADD and subsequent activation of caspase-8 (11). This caspase then catalyzes a series of proteolytic events that result in the characteristic biochemical and morphological changes associated with apoptosis.

In this study, we show that 2ME<sub>2</sub> induces apoptosis through engagement of the extrinsic pathway. 2ME<sub>2</sub> treatment of a variety of tumor cells as well as endothelial cells results in up-regulation of DR5 *in vitro* and *in vivo*. It also renders the cells more sensitive to the DR5 ligand TRAIL, which acts synergistically with 2ME<sub>2</sub> in inducing apoptosis *in vitro*. The findings that cell death induced by 2ME<sub>2</sub> requires the activation of caspases, and the early activation of caspase-8 before caspase-9 and caspase-3, are also consistent with 2ME<sub>2</sub> activation of the extrinsic pathway. Finally, blocking DR signaling by stable transfection of a DN-FADD construct results in severe impairment of the apoptotic response induced by 2ME<sub>2</sub>.

### MATERIALS AND METHODS

**Chemicals.** 2ME<sub>2</sub> was purchased from Tetriconics (Madison, WI) and 30 mM stock solutions were made in DMSO and stored in aliquots at  $-80^{\circ}\text{C}$ . The compound was diluted in incubation media immediately before each experiment. Thawed stock solutions were used once and discarded. The following reagents were purchased: soluble recombinant human TRAIL protein and anti-His antibody (R&D Systems, Minneapolis, MN or Alexis Biochemicals, San Diego, CA); TNF- $\alpha$  (R&D Systems); anti-Fas clone CH-11 (Upstate Biotechnology, Lake Placid, NY); zVAD-fmk (Alexis Biochemicals); and cycloheximide (Sigma, St. Louis, MO).

**Cell Culture.** Human breast carcinoma cells MDA-MB-231 and MDA-MB-435, cervical carcinoma cells HeLa, prostate carcinoma cells PC-3, and glioma cells U87-MG were maintained in DMEM:Ham's F-12 (1:1) medium containing 10% (v/v) fetal bovine serum (Hyclone Laboratories, Logan, UT) and 1 $\times$  antibiotic-antimycotic (BioWhittaker, Walkersville, MD).

HUVEC were obtained from Clonetics (San Diego, CA). HUVEC cultures were maintained for up to five passages in endothelial growth medium containing bovine brain extract (Clonetics) and 1 $\times$  antibiotic-antimycotic.

**Apoptosis Assays.** Apoptosis was measured by examination of the presence of cytoplasmic histone-associated DNA fragments. Briefly, cells were seeded in a 24-well plate and after 24 h were exposed to drug for the indicated times. Detection of cytoplasmic histone-associated DNA fragments was performed according to the manufacturer's instructions (Roche, Indianapolis, IN).

**Immunoblot Analysis.** Subconfluent monolayers of cells were treated with the indicated concentrations of 2ME<sub>2</sub>. Cellular supernatant was removed and collected, cells were scraped in cold PBS, and the total cellular content was collected by centrifugation. Cell pellets were lysed in 50  $\mu\text{l}$  of cold lysis buffer [10 mM Tris (pH 7.5) containing 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, 2  $\mu\text{g}/\text{ml}$  aprotinin, 2  $\mu\text{g}/\text{ml}$  leupeptin, and 1 mM sodium vanadate], and the lysate was clarified by centrifugation at  $4^{\circ}\text{C}$ . Protein concentration was determined by method of Bradford. The cytosol was mixed with an equal volume of NuPage LDS sample buffer (Invitrogen, Carlsbad, CA) containing 10% 2-mercapto-

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<sup>3</sup> The abbreviations used are: 2ME<sub>2</sub>, 2-methoxyestradiol; AFC, 7-amino-4-trifluoromethyl coumarin; DAPI, 4',6-diamidino-2-phenylindole; DN-FADD, dominant-negative Fas-associated death domain; DR, death receptor; ER, estrogen receptor; HUVEC, human umbilical vein endothelial cells; PARP, poly(ADP-ribose) polymerase; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand.

ethanol (Sigma) and heated at 95°C for 5 min. Equal protein concentrations of cytosol lysate were subjected to 4–20% Tris-glycine SDS-PAGE (precast NOVEX; Invitrogen). Subsequently, protein was electrophoretically transferred to nitrocellulose membranes and blotted with either a rabbit anti-DR5 polyclonal antibody (Cell Sciences, Inc., Norwood, MA) or a rabbit antiactin polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or a rabbit anti-PARP polyclonal antibody (Santa Cruz Biotechnology, Inc.). Proteins were visualized by the enhanced chemiluminescence detection system (Amersham, Piscataway, NJ) using a horseradish peroxidase-conjugated antibody against rabbit or mouse IgG (Bio-Rad, Hercules, CA).

**Immunofluorescence.** MDA-MB-231 cells were plated on chamber slides (35 × 10<sup>3</sup> cells/chamber, Falcon Culture Slides) and after 24 h were treated with media alone or containing 1 μM 2ME<sub>2</sub> for 24 h. Attached cells were fixed with 4% paraformaldehyde for 25 min at 4°C. Slides were then washed two times in PBS for 5 min. After incubation for 5 min on ice with 0.2% Triton X-100 in PBS, the grid housing was removed and slides were washed twice in PBS. Chamber slides were then stored at –20°C until analysis. Chamber slides were warmed to room temperature and rehydrated in dH<sub>2</sub>O for 20 min were blocked with 1% fish skin gelatin (Sigma), 0.2% Triton X-100 in PBS for 50 min, rinsed twice in PBS followed by Avidin and Biotin Block (Vector Laboratories, Burlingame, CA). The preparations were then incubated with 1:100 dilution of rabbit antiactive Caspase-3 monoclonal antibody (PharMingen, San Diego, CA) at 4°C overnight, rinsed three times sequentially in dH<sub>2</sub>O, PBS with gelatin/Triton X-100 and PBS, and finally incubated with biotinylated antirabbit secondary antibody for 40 min (1:200 dilution), followed by dark room incubation with Fluorescein Avidin D (1:100 dilution) 40 min (Vector Laboratories). Chamber slides were then incubated with 1% fish gelatin, 0.2% Triton X-100 in PBS for 50 min followed by a rabbit polyclonal anti-DR5 (1:100 dilution; Cell Sciences, Inc.) for 1.5 h in the dark at room temperature followed by direct conjugation with antirabbit Texas Red Alexia 594 for 30 min (Molecular Probes, Inc., Eugene, OR). After the final wash, the slides were mounted and stained with DAPI that produces blue fluorescence. Immunofluorescence microscopy was performed using a BX60 microscope (Olympus).

**Tumor Models and Immunohistochemistry.** Female 8–10 week-old BALB/c nu/nu mice (The Jackson Laboratory, Bar Harbor, ME) received injections in the mammary fat pad with 2 × 10<sup>6</sup> MDA-MB-231 cells. Daily oral administration of 2ME<sub>2</sub> was initiated when the tumors became palpable (4–5 mm diameter). A suspension of 2ME<sub>2</sub> was prepared by either homogenization in an aqueous solution of 0.5% methylcellulose (Sigma) using a Kinematica polytron (Brinkmann, Westbury, NY), or 2ME<sub>2</sub> was incorporated into liposomes composed of dioleoyl phosphatidylcholine with a molar ratio of phospholipid:2ME<sub>2</sub> of 2:1.5. Control animals were given vehicle alone or liposomes composed of dioleoyl phosphatidylcholine alone. Tumor volumes were calculated as width<sup>2</sup> × length × 0.52. Upon termination of the dosing regimen, animals were euthanized with CO<sub>2</sub>, and tumors were excised and fixed in 10% buffered formalin. Paraffin-embedded tissue samples were sectioned (5 μm) and mounted on Superfrost slides (Fisher Scientific, Co., Houston, TX). Sections were deparaffinized in xylene, subsequently immersed in graded alcohols (100%, 95% ethanol) and rehydrated in dH<sub>2</sub>O. Sections were then treated with EDTA buffer for heat-induced epitope retrieval (NeoMarkers, Inc., Union City, CA). Samples were washed twice in dH<sub>2</sub>O and blocked for endogenous peroxidase activity by use of 3% hydrogen peroxide in methanol for 20 min. After washing sections in dH<sub>2</sub>O, samples were incubated with 5% normal rabbit serum for 40 min and then incubated with the primary antibody, polyclonal goat anti-DR5 antibody (1:100 dilution; Alexis Biochemicals), for 18 h at 4°C. Slides were rinsed in dH<sub>2</sub>O, washed twice in PBS, incubated with biotinylated antigoat secondary antibody (1:200 dilution), washed twice in PBS, and finally incubated with Elite ABC Peroxidase Kit (Vector Laboratories). After a final wash in PBS, positive reaction was visualized by incubating the slides with 3,3'-diaminobenzidine for 5 min (Vector Laboratories). The slides were then rinsed in dH<sub>2</sub>O, counterstained with Gills Hemotoxylin III (Sigma), rinsed, and immersed briefly in 1% ammonia solution for nuclei binding. Slides were hydrated and mounted with cytoqual (VWR International, West Chester, PA). For negative controls, the same procedure was performed with the omission of either the primary or secondary antibody.

**Measurement of Caspase Enzymatic Activity.** Caspase activation was measured using a caspase fluorometric protease assay kit as described by the

manufacturer (BioVision Research Products, Palo Alto, CA). Briefly, after treatment of cells with 2 μM 2ME<sub>2</sub> for the indicated periods of time, cells were harvested, pelleted, and frozen on dry ice. Cell lysis buffer was added to the cell pellets, and protein concentration was determined by using Bradford analysis. A total of 200 μg of cell lysates was incubated with 5 μl of a 1 mM stock of the respective fluorescently labeled tetrapeptide at 37°C for 1 h. The release of AFC was measured with a fluorometer (Wallac Victor; Perkin-Elmer Life Sciences, Boston, MA) at an excitation wavelength of 400 nm and an emission wavelength of 505 nm. Caspase activities were assayed in duplicate samples. Caspase-8-, caspase-9-, and caspase-3-like enzymatic activities were assessed in parallel.

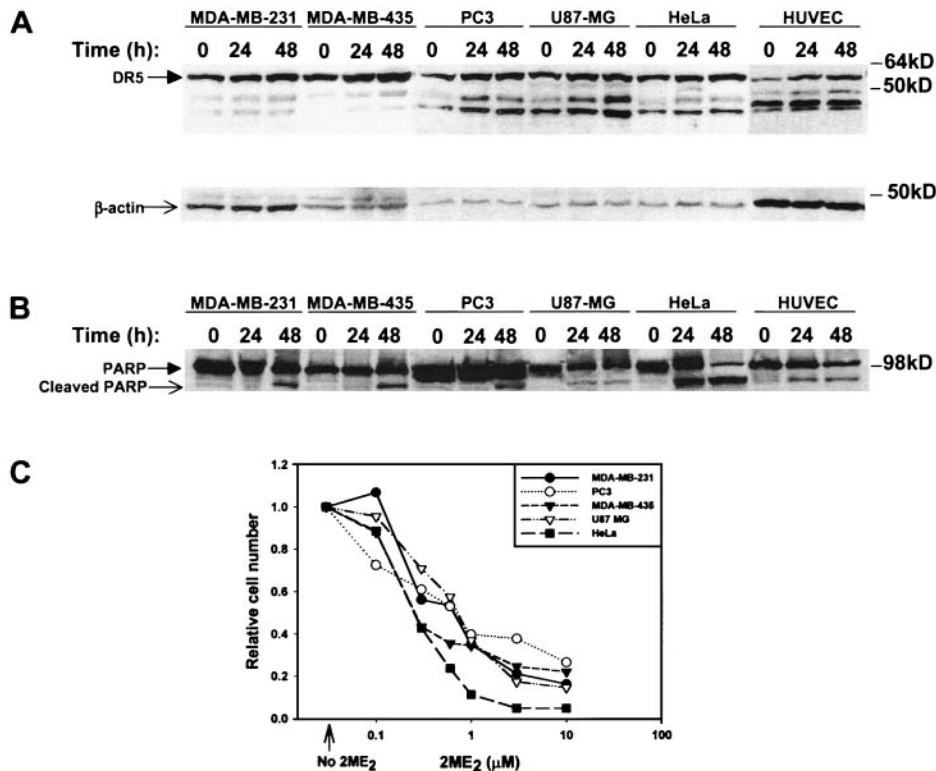
**Stable Transfection.** The expression vector pcDNA3-FADD-DN [kind gift of Dr. Mark Williams (American Red Cross)], which carries the cDNA insert of human DN-FADD under the control of the cytomegalovirus promoter or the vector pcDNA3.1 (Promega) without insert, were used to generate stable transfectants. MDA-MB-231 cells were plated at 5 × 10<sup>5</sup> cells/10-cm dish, and the next day were transfected with 1 μg of plasmid using the SuperFect reagent (Qiagen, Valencia, CA) according to the manufacturer's instructions. Resistant colonies were selected in 2 mg/ml G418. Ten isolated colonies were selected, and positive clones expressing the DN-FADD construct were verified by Western blot analysis using an anti-FADD monoclonal antibody (Transduction Laboratories, Lexington, KY).

## RESULTS

**2ME<sub>2</sub> Treatment *in Vitro* Induces DR5 Up-Regulation and Apoptosis in a Variety of Cell Types.** It is well established that 2ME<sub>2</sub> induces apoptosis in tumor cells and that it also has antiangiogenic activity. 2ME<sub>2</sub> inhibits several stages in the angiogenic cascade, including inhibition of proliferation and induction of apoptosis in proliferating endothelial cells (reviewed in Refs. 12, 13). In an effort to define the signaling pathways used by 2ME<sub>2</sub>, a preliminary study with a cDNA array and mRNA from ECV cells treated with 2ME<sub>2</sub> revealed that DR5 mRNA was up-regulated (data not shown). To determine whether induction of DR5 protein was a general response of cells exposed to 2ME<sub>2</sub>, we analyzed whole cell lysates of either untreated or 2ME<sub>2</sub>-treated cells by immunoblotting. We found that DR5 protein expression is induced in a variety of tumor cell types and in HUVEC primary endothelial cell type (Fig. 1A). The appearance of multiple polypeptides in the DR5 immunoblot has been reported previously (14, 15). This up-regulation is associated with an apoptotic response, as indicated by the appearance in 2ME<sub>2</sub>-treated cells of the M<sub>r</sub> 85,000 PARP cleavage fragment (Fig. 1B). The IC<sub>50</sub> values for the antiproliferative activity of 2ME<sub>2</sub> are in a narrow range from high nanomolar to low micromolar, and consequently, a 2-μM concentration was selected to evaluate the effects of 2ME<sub>2</sub> on the various cell types. However, with HeLa cells, we used a concentration of 1 μM because in these cells the overall cell death is greater, although the IC<sub>50</sub> is not particularly different from other cell types. Treatment of HeLa cells with 1 μM 2ME<sub>2</sub> for 48 h results in an 88.5% decrease in cell number, whereas treatment of the other cell lines with 1 μM 2ME<sub>2</sub> results in only an approximate 65% decrease (Fig. 1C). It is interesting that the appearance of the PARP cleavage fragment varies among cell lines. In MDA-MB-231, MDA-MB-435, and PC3 cells, 2ME<sub>2</sub> treatment results in the appearance of the PARP cleavage fragment at 48 h, whereas in HUVEC and U87-MG cells, appearance of detectable level of PARP cleavage fragment occurs at 24 h and does not increase with additional treatment. Consistent with HeLa cells being more responsive to 2ME<sub>2</sub>, the greatest extent of PARP cleavage in response to treatment occurs with these cells (Fig. 1B).

We also assessed other DRs. Although 2ME<sub>2</sub> treatment did result in up-regulation of DR4 protein in PC3 cells, this up-regulation was not a general response to 2ME<sub>2</sub> in all of the tumor cell lines that we assessed. Consistent with previous reports, in endothelial cells we found up-regulation of Fas in response to 2ME<sub>2</sub> treatment in HUVEC

Fig. 1. 2ME<sub>2</sub> induces up-regulation of DR5 and apoptosis in a variety of cell types. Cell lines were either left untreated or treated with 2ME<sub>2</sub> for the indicated times, culture media and cells were collected together, whole cell lysates were made, and immunoblot analysis was performed. *A*, DR5 immunoblot (*top panel*). The same blots were subsequently probed with anti- $\beta$ -actin (*bottom panel*) to demonstrate comparable protein loading. The arrow indicates the 55 kDa DR5 polypeptide (*top panel*) or the 45 kDa actin polypeptide (*bottom panel*). Densitometry analysis: MDA-MB-231 1.18 and 1.35, MDA-MB-435 1.36 and 1.38, PC3 2.50 and 2.47, U87-MG 1.53 and 1.34, HeLa 0.84 and 1.22, and HUVEC 2.41 and 1.60, indicating fold increase over the untreated control at 24 and 48 h, respectively. *B*, PARP immunoblot analysis. The closed arrow denotes the 116 kDa full-length PARP polypeptide, and the  $\rightarrow$  indicates the cleaved 85 kDa polypeptide fragment. The figure is a composite of three separate gels. *C*, to determine the antiproliferative activity, cells were treated with the increasing concentrations of 2ME<sub>2</sub> for 48 h, and proliferation was determined by cell counts. The IC<sub>50</sub>s for the various cell lines are 0.67  $\mu$ M (MDA-MB-231), 0.70  $\mu$ M (PC3), 0.27  $\mu$ M (MDA-MB-435), 0.50  $\mu$ M (U87-MG), and 0.27  $\mu$ M (HeLa).



(16). However, 2ME<sub>2</sub> had no effect on Fas in tumor cell lines. Similarly, up-regulation of TRAIL was observed in 2ME<sub>2</sub> treated HUVEC but not in tumor cell lines (data not shown).

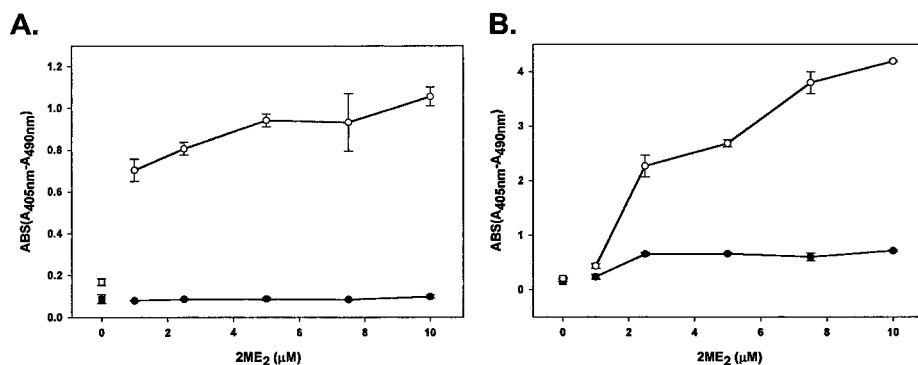
**2ME<sub>2</sub> Treatment Sensitizes MDA-MB-231 Cells and HUVEC to TRAIL-induced Apoptosis.** TRAIL, the ligand for DR5, has cytotoxic activity on a wide variety of transformed cell lines *in vitro* and is a potent antitumor agent *in vivo* (17, 18). However, up-regulation of DR5 does not always predict susceptibility to TRAIL-induced apoptosis (19–21). Because 2ME<sub>2</sub> up-regulates DR5, we investigated whether 2ME<sub>2</sub> treatment sensitizes tumor and endothelial cells to TRAIL-induced apoptosis. Treatment of either MDA-MB-231 cells or HUVEC with 50 ng/ml TRAIL alone or 2ME<sub>2</sub> alone induces apoptosis. Exposure of both cell types to TRAIL in combination with 2ME<sub>2</sub> results in a marked increase in apoptosis as compared with either treatment alone (Fig. 2, *A* and *B*). The strong induction of apoptosis by the combination treatment makes it appear that 2ME<sub>2</sub> alone does not significantly induce apoptosis. However, with more prolonged incubation during the color development of the ELISA, a dose-dependent increase in apoptosis in response to 2ME<sub>2</sub> is observed (Fig. 3). These data are consistent with the DR5 expressed in these

cells being able to induce apoptosis and indicate that 2ME<sub>2</sub> treatment further sensitizes cells to TRAIL-induced cell death.

**2ME<sub>2</sub>-induced Caspase Activation and Kinetics Are Consistent with DR-mediated Apoptosis.** DR5 activated apoptosis has been shown to be mediated via a caspase signaling cascade in which caspase-8, an initiator caspase, becomes activated and, in turn, activates other initiator caspases such as caspase-9 followed by activation of executioner caspases such as caspase-3 (11). To determine whether caspase activation is required for 2ME<sub>2</sub>-induced cell death, we assessed the ability of 2ME<sub>2</sub> to cause apoptosis in the presence of zVAD-fmk, a broad specificity caspase inhibitor. MDA-MB-231 cells or HUVEC pretreated with 50  $\mu$ M zVAD-fmk were incubated for 48 h with increasing concentrations of 2ME<sub>2</sub> or without the inhibitor, and the levels of apoptosis were assessed. As shown in Fig. 3, 2ME<sub>2</sub>-induced apoptosis in MDA-MB-231 cells and HUVEC was greatly attenuated in the presence of zVAD-fmk. These data suggest that both in tumor and endothelial cells 2ME<sub>2</sub> requires the activation of caspases to induce apoptosis.

To further characterize the apoptotic pathway activated by 2ME<sub>2</sub>, we determined the kinetics of caspase activation. The activation of

Fig. 2. Apoptosis assay with 2ME<sub>2</sub> and TRAIL. (*A*) MDA-MB-231 cells and (*B*) HUVECs were treated with the indicated concentrations of 2ME<sub>2</sub> in the absence (●) or presence (○) of 50 ng/ml TRAIL for 24 h, and apoptosis was determined by the cell death detection ELISA (Roche). The results are presented as the average of triplicates  $\pm$  SD.





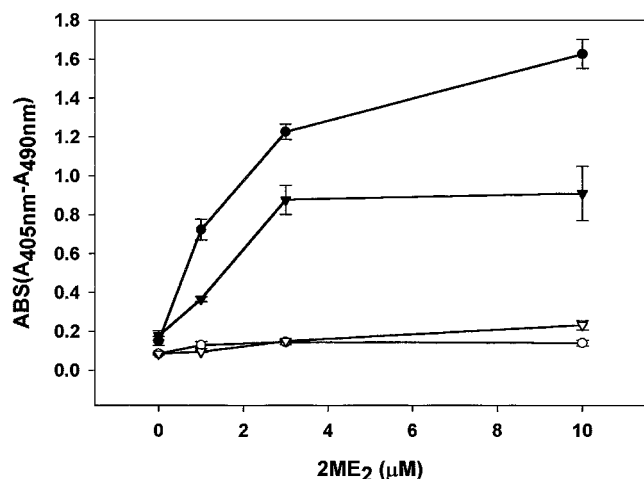


Fig. 3. 2ME<sub>2</sub> requires caspase activation for induction of apoptosis. MDA-MB-231 cells (circles) and HUVEC (triangles) were treated with increasing concentrations of 2ME<sub>2</sub> in the absence (● or ▼, respectively) or presence (○ or ▽, respectively) of 50 µM zVAD-fmk for 48 h, and apoptosis was determined using the cell death detection ELISA (Roche). The results are presented as the average of triplicate samples ± SD.

caspases in 2ME<sub>2</sub>-treated cells was assessed using the fluorogenic tetrapeptide substrates, IETD-AFC, LEHD-AFC and DEVD-AFC, which have been shown to be selective for caspase-8-, caspase-9-, and caspase-3-like enzymatic activities, respectively. Treatment of MDA-MB-231 cells with 2 µM 2ME<sub>2</sub> results in the detection of caspase-8 activation as early as 2 h followed by caspase-9 activation at 4 h and caspase-3 activation at 8–14 h (Fig. 4). The level of caspase-8 and caspase-9 activation increased up to 16 h of treatment. Caspase-3 activation continued to intensify over time and resulted in a much greater fluorescence signal than either caspase-8 or caspase-9 (Fig. 4). Our observation that caspase-8 is the first caspase activated by 2ME<sub>2</sub> is consistent with an apoptotic pathway initiated by DR activation.

**2ME<sub>2</sub> Activates Caspase-3 in Cells that Up-Regulate DR5.** To identify whether there is a relationship between DR and caspase activation, we assessed the up-regulation of DR5 and the activation of caspase-3 by immunofluorescence in cultured cells. MDA-MB-231 cells were either left untreated or treated with 1 µM 2ME<sub>2</sub> for 24 h, the adherent cells were fixed, and nuclear morphology was visualized by staining the cells with DAPI (Fig. 5). A slightly lower concentration of 2ME<sub>2</sub> was used in these experiments as compared with our immunoblot analysis (Fig. 1, A and B) to increase the number of cells retained on the coverslip, however, similar results were obtained with 2 µM 2ME<sub>2</sub>. In the treated population, there is a clear decrease in the number of cells and an increase in the number of mitotic figures and condensed nuclei. Cells stained with anti-DR5 antibody followed by a rodamine-labeled secondary antibody (Fig. 5) showed brighter staining of the treated population overall consistent with the increase in DR5 protein expression observed by immunoblot analysis (Fig. 1A). Staining with an antibody that recognizes the activated cleavage fragment of caspase-3 followed by a FITC-labeled secondary antibody shows that despite occasional staining of cells in the untreated population, there are significantly more activated caspase-3-positive cells in the treated cells. Because in this assay only the cells that are still attached were evaluated, this assessment underestimates the total number of cells that have activated caspase-3. Costaining with anti-DR5 and antiactivated caspase-3 antibodies shows that 2ME<sub>2</sub> treatment results in active caspase-3 only in cells that have up-regulated DR5 as indicated by the yellow cells (Fig. 5), consistent with DR5-dependent caspase-3 activation.

**DN-FADD Inhibits 2ME<sub>2</sub>-induced Apoptosis.** Several DRs signal apoptosis by recruitment of the adaptor protein FADD to the

oligomerized DR complex, where FADD facilitates the binding and activation of procaspase-8. To further test the hypothesis that DR5 mediates 2ME<sub>2</sub>-induced apoptosis, DN-FADD was stably transfected into MDA-MB-231 cells. As shown in Fig. 6A, Fas, TNF, and TRAIL-mediated apoptosis were markedly reduced by stable overexpression of DN-FADD protein as compared with the treated cells transfected with vector alone. These death-inducing cytokines have been shown to mediate apoptosis through a DR-mediated pathway that activates caspase-8 through a FADD-dependent pathway (22, 23). Similarly, 2ME<sub>2</sub> treatment of two independent clones of cells transfected with DN-FADD resulted in a marked reduction in the amount of apoptosis observed as compared with the cells expressing the vector alone (Fig. 6B). These data indicate that a DR-mediated pathway is used in the mechanism of 2ME<sub>2</sub>-induced apoptosis. Although these data do not exclude a role of other DRs, only DR5 is up-regulated in these cells in response to 2ME<sub>2</sub> treatment.

**p.o. Administered 2ME<sub>2</sub> Inhibits Primary Tumor Growth and Up-Regulates DR5 *in Vivo*.** 2ME<sub>2</sub> has been shown to be effective at inhibiting both primary tumors and metastatic disease in a variety of

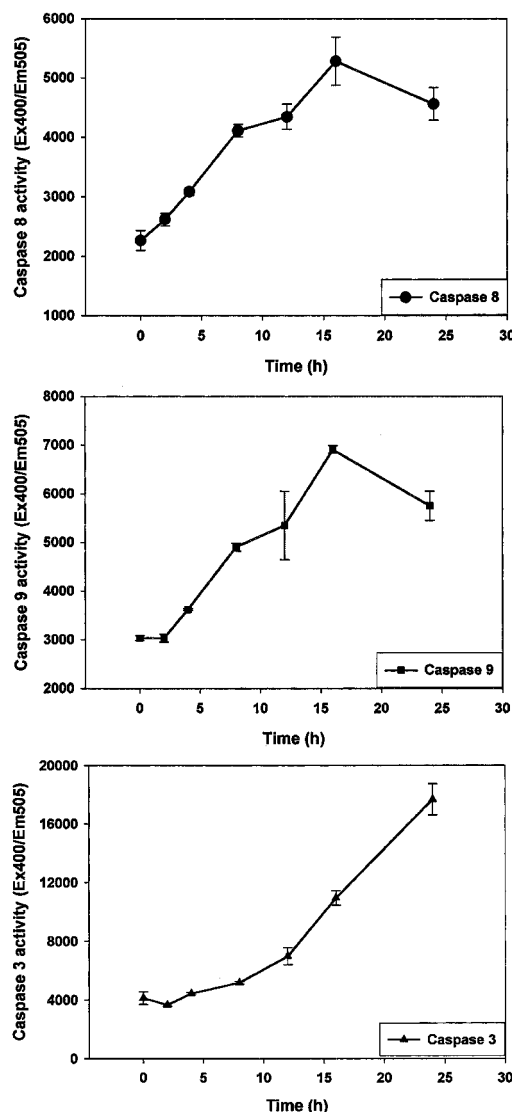


Fig. 4. Kinetics of 2ME<sub>2</sub>-induced caspase activation. MDA-MB-231 cells were treated with 2 µM 2ME<sub>2</sub> for the indicated times, cell lysates were made, and caspase-8-, caspase-9-, and caspase-3-like enzymatic activities were measured by hydrolysis of IETD-AFC, LEHD-AFC, and DEVD-AFC, respectively, using a fluorescence plate reader. The results are presented as the average ± SD.

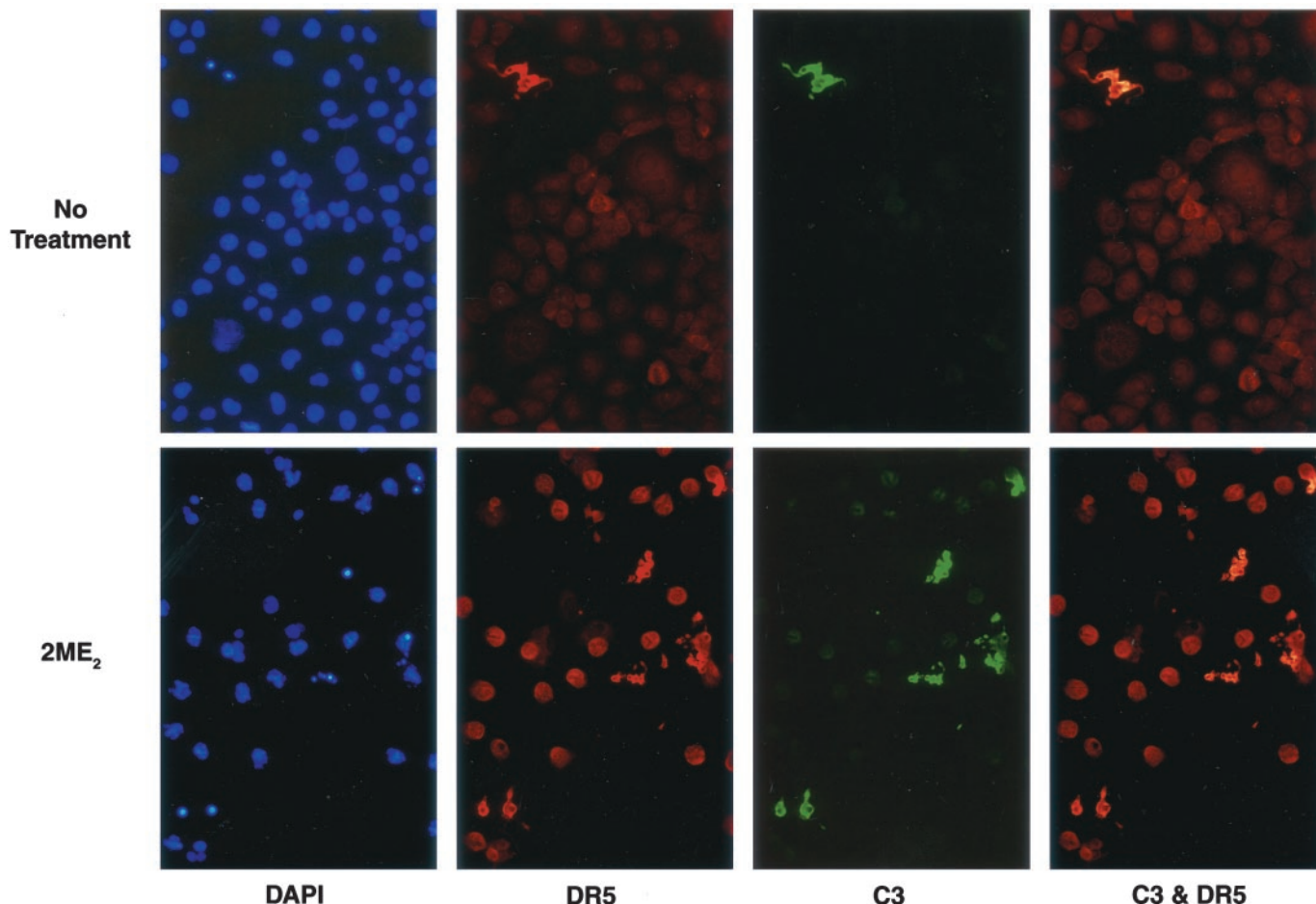


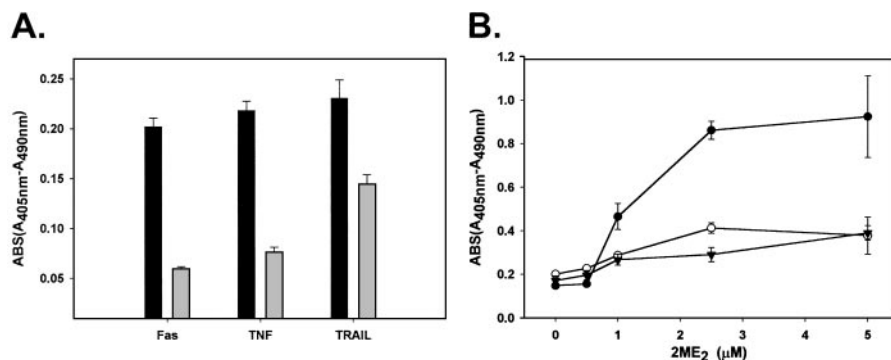
Fig. 5. Colocalization of activated caspase-3 with up-regulated DR5 in 2ME<sub>2</sub>-treated cells. MDA-MB-231 cells were plated on chamber slides untreated or treated with 1  $\mu$ M 2ME<sub>2</sub> for 24 h, fixed with 4% paraformaldehyde (methanol free) and incubated with 0.2% Triton X100. Nuclear morphology was visualized in MDA-MB-231 cells using DAPI stain (blue). Immunofluorescence double labeling with antibodies against DR5 (red, labeled with Texas Red) or activated caspase-3 (green, labeled with FITC). Colocalization of DR5 and activated caspase-3 are visible as yellow staining generated by merging the color images. Each of the respective images was taken using identical exposures.

tumor models through induction of apoptosis (12, 13). Oral administration of 2ME<sub>2</sub> in an orthotopic MDA-MB-231 breast cancer model in nude mice resulted in a reduction of tumor volume (Fig. 7A). At the termination of study the tumor burden was reduced ~65% in the animals treated with 150 mg/kg 2ME<sub>2</sub>. Consistent with data using cultured cells, immunohistochemistry analysis of MDA-MB-231 tumors demonstrated that 2ME<sub>2</sub> treatment results in up-regulation of DR5 protein expression (Fig. 7B) but not DR4 protein expression (data not shown). Similar results were obtained using U87-MG in a xenograft tumor model (24). These data show that treatment of tumors with doses of 2ME<sub>2</sub> that produce a reduction in tumor burden in two animal models results in the up-regulation of DR5 protein.

## DISCUSSION

2ME<sub>2</sub> is an endogenous metabolite of estradiol, yet it is distinct among estradiol metabolites because of its inability to engage ERs as an agonist. In addition, its unique antiproliferative and apoptotic activities are mediated independently of the ERs (3). 2ME<sub>2</sub> is also unique among the many antiangiogenic agents currently in clinical trials because it targets both the endothelial cell and the tumor cell compartment of a growing tumor. The physiological function and molecular mechanism of this compound are not clearly defined. To further understand the mechanism of action of 2ME<sub>2</sub>, we investigated the pathways of activation of the apoptotic response. In this study, we

Fig. 6. DN-FADD attenuates 2ME<sub>2</sub>-induced apoptosis. A, MDA-MB-231 cells transfected with vector alone (■) or with DN-FADD expression vector (□) were treated with 100 ng/ml anti-Fas IgM (clone CH-11) or 100 ng/ml TNF $\alpha$  in the presence of cycloheximide or with 1 ng/ml TRAIL + 1  $\mu$ g/ml anti-his antibody for 24 h. B, MDA-MB-231 cells transfected with vector alone (●) or with DN-FADD expression vector clone 2 (○) and clone 4 (▼) were treated with increasing concentrations of 2ME<sub>2</sub> for 24 h, and apoptosis was determined using the cell death detection ELISA (Roche). The results are presented as the average of triplicates  $\pm$  SD.



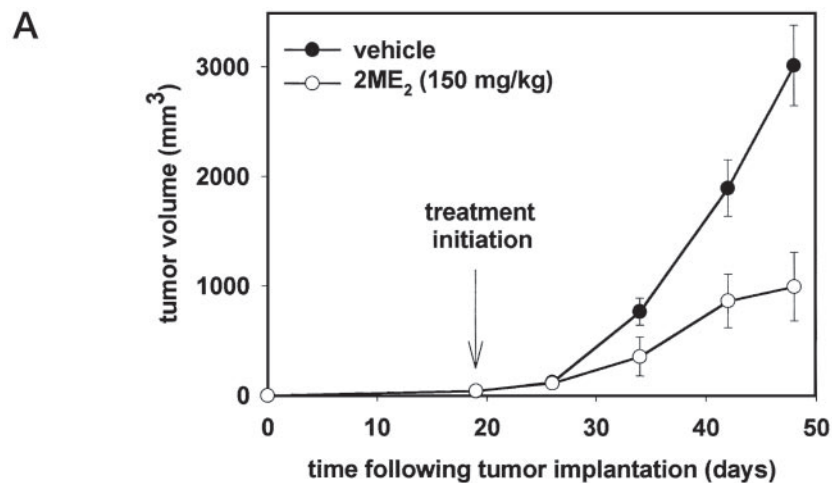
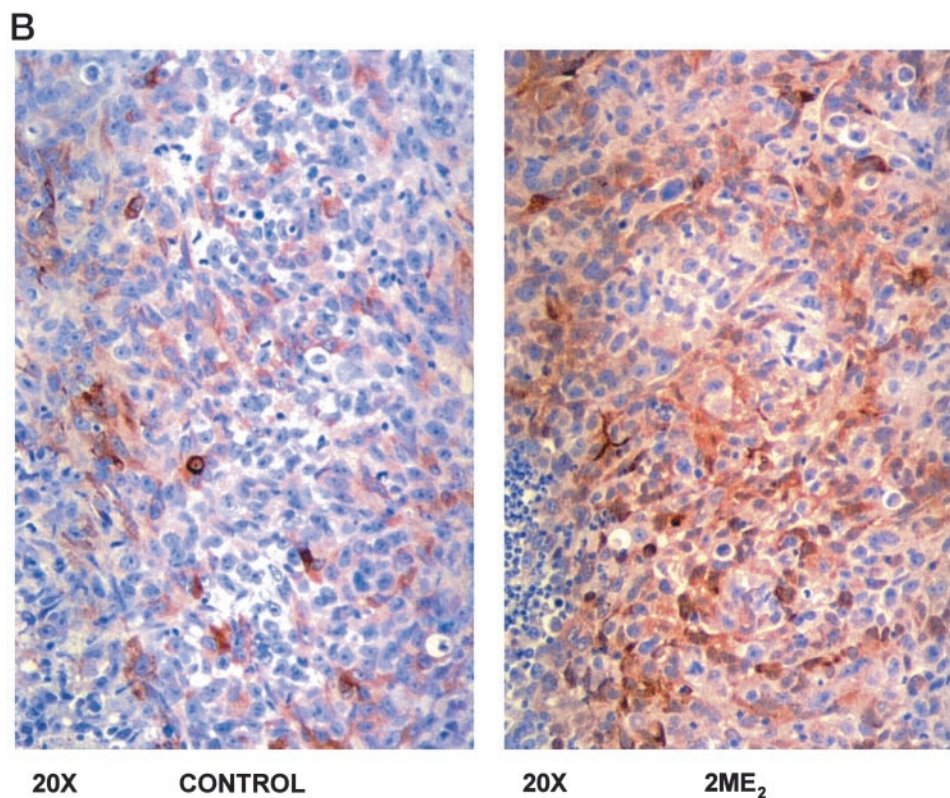


Fig. 7. 2ME<sub>2</sub> induces increased expression of DR5 protein *in vivo*. *A*, tumors were established using human MDA-231-MB cells ( $2 \times 10^6$ ) implanted into the mammary fat pad of female BALB/c nu/nu mice. Oral administration of a 2ME<sub>2</sub> suspension in 0.5% methylcellulose was initiated at the indicated times, and tumor measurements were calculated using the formula: width<sup>2</sup>  $\times$  length  $\times$  0.52. Mean  $\pm$  SE ( $n = 6$ ) of representative experiments is shown. *B*, human orthotopic MDA-MB-231 tumors were removed and fixed in 10% buffered formalin. Tissue was processed and embedded in paraffin, and the sections were immunostained for expression of DR5 using goat polyclonal antisera (Alexis Corp) and Vectastain Elite ABC kit peroxidase (Vector). The peroxidase was visualized with 3,3'-diaminobenzidine and then counterstained with hematoxylin. A  $\times 20$  exposure is shown.



show that 2ME<sub>2</sub> treatment results in an increase in DR5 protein expression in cultured cells at doses that induce apoptosis and in tumor models at doses that produce a significant decrease in tumor burden. 2ME<sub>2</sub> treatment of cells results in the activation of caspase-8 followed by caspase-9 and caspase-3; furthermore, caspase-3 activation was identified only in 2ME<sub>2</sub>-treated cells that have up-regulated DR5 protein. Finally, 2ME<sub>2</sub>-induced apoptosis is attenuated by over-expression of DN-FADD. Collectively, these data show that 2ME<sub>2</sub> mediates apoptosis through engagement of the extrinsic pathway.

In our evaluation of which DRs were induced in response to 2ME<sub>2</sub> treatment, we found that DR5 was consistently up-regulated. DR4 protein up-regulation was observed in treated PC3 cells *in vitro*; however, there was no significant increase in DR4 protein expression in response to 2ME<sub>2</sub> treatment *in vivo*. Consistent with previous reports, 2ME<sub>2</sub> treatment resulted in up-regulation of Fas in HUVEC, but this treatment did not result in up-regulation of Fas in tumor cell lines (data not shown). Similarly, the ligand for DR5 TRAIL was

up-regulated in response to treatment of HUVEC but not of tumor cell lines. Our *in vivo* analysis showed a mixed response in that only some of the treated animals showed a slight increase of TRAIL protein expression. Thus, whereas DR4, Fas, and TRAIL up-regulation varied as a function of cell type, DR5 was consistently up-regulated in response to 2ME<sub>2</sub> treatment. Alternatively, TRAIL produced in the tumor vasculature could exert its effects in the surrounding tumor cells. Currently, this cannot be assessed in these models because antibodies recognizing murine TRAIL are not available.

The cell type-specific Fas and TRAIL up-regulation suggest that some aspects of the mechanisms by which 2ME<sub>2</sub> induces apoptosis in tumor and endothelial cells may be distinct. Two antiangiogenic proteins, pigment epithelium-derived factor and thrombospondin 1, have recently been reported to selectively induce apoptosis in proliferating endothelial cells by up-regulating FAS ligand. Fas was shown to be up-regulated in only proliferating endothelial cells and, thus, allows these antiangiogenic agents to selectively inhibit the growing



or remodeling endothelium (25). Whether these endogenous angiogenesis inhibitors also up-regulate DR5 is unknown. The finding that several 2ME<sub>2</sub> analogs show preferential inhibition of endothelial cells over tumor cells (26, 27) additionally suggests that the mechanisms of 2ME<sub>2</sub>-induced apoptosis in endothelial cells *versus* tumor cells may be different.

There are several possible mechanisms by which 2ME<sub>2</sub>-induced apoptosis in tumor cell lines *in vitro* could be mediated through DR5. TRAIL protein is present and it is possible, as has been suggested for other agents that up-regulate DR5 without up-regulating TRAIL, that the newly generated DR is able to use the constitutively expressed ligand. However, we were unable to affect 2ME<sub>2</sub>-induced apoptosis with a soluble DR5 alone or in combination with an antagonist antibody against Fas and soluble TNF receptor (data not shown). These data suggest either that the interaction with the endogenous ligand is not readily accessible to exogenously added inhibitors or that the up-regulated DR5 does not require additional activation. This has been shown in transfected cell lines in which overexpression of DR5 can induce apoptosis in a ligand-independent manner (23). The up-regulation of DR5, the kinetics of caspase activation, the coexpression of activated caspase-3 only in cells expressing DR5, and the attenuation of apoptosis mediated by expression of DN-FADD are all consistent with DR5 involvement in 2ME<sub>2</sub>-mediated cell death, irrespective of the mechanism underlying this process.

The inhibitory effects of 2ME<sub>2</sub> have been attributed to several mechanisms, including disruption of microtubule function (4–6), inhibition of superoxide dismutase (9), inhibition of angiogenesis (28), and induction of apoptosis (16, 29). 2ME<sub>2</sub> has been demonstrated to cause G<sub>2</sub>-M arrest, to bind to tubulin and microtubules inhibiting both polymerization and depolymerization (4, 5), and to cause abnormal spindle formation in cultured cells (30). In contrast, it has been also reported that treatment of cells with antiproliferative concentrations of 2ME<sub>2</sub> cause microtubule stabilization in some cases (6) and does not result in inhibition of mitotic spindles in others (31). We have only observed destabilization of microtubules on cells in culture at concentrations of 2ME<sub>2</sub> significantly higher than those used in this study to see effects on DR5 up-regulation. However, effects on microtubule dynamics occur at concentrations that do not affect stability (32). We found that treatment of tumor-bearing animals with vinblastine, a drug that destabilizes microtubules, also resulted in up-regulation of DR5 protein (data not shown). Paclitaxel and docetaxel, two agents that stabilize microtubules, have been shown to up-regulate DR4 and DR5 protein expression and sensitize prostate cell lines to the cytotoxic effects of TRAIL (33). However, these effects may not represent a general cellular observation because paclitaxel did not up-regulate DR4 and DR5 protein or enhance the activity of TRAIL in non-small cell lung cancer or breast cancer cell lines (34–36). In the case of 2ME<sub>2</sub> it is still uncertain how up-regulation of DR5 is related to its effects on microtubule stability and this requires additional investigation.

In the current study, we demonstrate that 2ME<sub>2</sub> up-regulates DR5, uses the extrinsic apoptotic pathway, and makes cells more sensitive to TRAIL. Similar to 2ME<sub>2</sub>, TRAIL exerts potent antitumor activity *in vivo* without affecting normal tissue, suggesting that a combination of 2ME<sub>2</sub> and TRAIL may be an effective antitumor therapy. Furthermore, in the absence of dose-limiting toxicity for 2ME<sub>2</sub>, DR5 expression may serve as a surrogate marker for biological response in the clinic. DR5 may also serve as a marker for assessing and discriminating 2ME<sub>2</sub>-like molecules from other related antiproliferative agents because structurally related molecules could induce antiproliferative responses through a variety of mechanisms.

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