Inhibition of Normal and Experimental Angiotumor Endothelial Cell Proliferation and Cell Cycle Progression by 2-Methoxyestradiol (44334)

F. REISER,[1] D. WAY, M. BERNAS, M. WITTE, AND C. WITTE
Department of Surgery, The University of Arizona, Tucson, Arizona 85724

Abstract. **With rapid growth and metabolism, aggressive cancers require an extensive vascular network, termed tumor angiogenesis. The body produces a variety of natural angiogenic inhibitors, among which is the mammalian estrogen metabolite, 2-methoxyestradiol (2-MeOE$_2$). In this study, we compared the effects of 2-MeOE$_2$ on a human umbilical vein cell line (HUVEC-C) and on an immortal, angiotumor-producing rat sinusoidal endothelial cell line (RSE-1). *In vitro,* the effects of varying concentrations of 2-MeOE$_2$ from 0.01–100.0 μ*M* were measured with cell counts and compared to control cells. HUVEC-C had an ED$_{50}$ ~3.5 μ*M* with ~27% inhibition of cell growth whereas RSE-1 had an ED$_{50}$ ~2.2 μ*M* with ~50% inhibition of cell growth compared with controls. The lowest concentration with maximal effect was 10.0 μ*M* 2-MeOE$_2$ for both cell lines. Using this concentration, flow cytometric analysis of cell cycles was performed with propidium iodide stained DNA of HUVEC-C and RSE-1 at 24 and 48 hr. Both demonstrated a significant ($P < 0.0001$) block at G$_2$M of the cell cycle. At 48 hr, HUVEC-C had 32% of cells in G$_2$M (control = 9% G$_2$M), and RSE-1 had 36% of cells in G$_2$M (control = 18% G$_2$M). These findings demonstrate a strong *in vitro* antiproliferative effect of 2-MeOE$_2$ on normal dividing endothelial as well as angiotumor cells mediated through a cell cycle–specific block at G$_2$M. The antiendothelial, antiangiotumor effect of 2-MeOE$_2$ supports its potential as a therapeutic agent against solid organ cancers, benign or malignant vascular growths, and other pathologic states dependent on angiogenesis.** [P.S.E.B.M. 1998, Vol 219]

Aggressive tumors require an extensive neovascular network for rapid growth and metabolism, a phenomenon referred to as tumor angiogenesis. Folkman describes an initial, slow tumor growth phase that precedes vascular development, and tumor cells form a tiny ball of limited growth potential relying only on paracrine/autocrine factors and nutrient diffusion for growth stimulation (1). According to this concept, tumors remain in a dormant state until a blood supply is established. Once the tumor has induced surrounding blood vessels to supply nutrients and growth factors, it then has the ability to grow and metastasize with malignant potential. Tumor conversion to an angiogenic phenotype is a key step in malignancy and involves a balance between angiogenic stimulators and inhibitors secreted by the tumor and host. Thus, both tumor growth and metastasis are viewed as angiogenesis dependent. Angiogenesis not only enhances tumor cell growth but augments endothelial cell proliferation from a dormant cell type without mitotic figures to one of the most rapidly proliferating cell types in the body (2).

Angiogenesis is also critical in several other physiologic and pathologic states. For example, it enables the normal physiologic process of the menstrual cycle for reproduction, the inflammatory response for host protection,

This work was supported in part by medical student research fellowships (FR) from the American Heart Association (AHA 85-509), the National Institutes of Health (T35 HL07479), and the John J. and Lilian Bilyu Banchi Scholarship Trust of the University Heart Center and by a grant from the National Institutes of Health (HL R01HL48493) (MHW).

[1] To whom requests for reprints should be addressed at Lymphology Laboratories, Room 4406, Department of Surgery, University of Arizona, P.O. Box 245063, Tucson, AZ 85724. E-mail: *reiser@azstarnet.com*

Received June 1, 1998. [P.S.E.B.M. 1998, Vol 219]
Accepted August 11, 1998.

0037-9727/98/2193-0211$10.50/0

and wound healing, all necessary to sustain life (1–2). On the other hand, pathologic states such as diabetic retinopathy, rheumatoid arthritis, psoriasis, peptic ulcer disease, vasculitides and eczema also involve exuberant angiogenesis (1–2).

Expansion of the vascular network of a tumor depends on elaboration of host and tumor angiogenic factors. In addition to angiostimulatory factors, an array of inhibitors of angiogenesis have been identified that prevent tumor growth and metastasis by interrupting the development of a nutritive vascular network. Accordingly, several natural and synthetic antiangiogenic agents with different mechanisms of action are under active development for therapeutic use (1, 3, 4).

The natural mammalian estrogen metabolite, 2-methoxyestradiol, inhibits the proliferation of tumor and endothelial cells both *in vitro* and *in vivo*. To explore its mechanism of action and expand on these observations, we investigated the *in vitro* effects of 2-methoxyestradiol on proliferation and cell cycle events in normal endothelial cells (HUVEC-C) and in an angiotumor-producing, immortal cell line (RSE-1) (5) by examining cell counts and analyzing flow cytometeric cell cycle measurements.

Materials and Methods

Chemicals and Materials. 2-Methoxyestradiol (2-$MeOE_2$), dimethyl sulfoxide (DMSO), Dulbecco's modified eagle medium: Ham's F-12 (M5), heparin, endothelial cell growth supplement (ECGS), propidium iodide, and Nonidet P-40 were supplied by Sigma Chemical Co. (St. Louis, MO). HUVEC-C cells (ATCC CRL-1730) were supplied by the American Type Culture Collection (ATCC) (Rockville, MD). RSE-1 is an immortal cell line [The University of Arizona, Department of Surgery, Lymphology Laboratory] derived from an isolate of rat sinusoidal endothelium.

Cell Cultures. RSE-1 cells were routinely grown in M5 medium (1:1 DMEM:Ham's F-12) supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/ml)-streptomycin (100 μg/ml), and fungizone (2.5 μg/ml). HUVEC-C cells were routinely grown in the previously described M5 medium supplemented additionally with 50 μg/ml ECGS and 100 μg/ml heparin. Flasks for HUVEC-C were coated with gelatin by first coating the flask with a small amount of 0.2% gelatin in distilled H_2O followed by heating the surface to attach and dry the gelatin coating.

Dose-Response Experiments. RSE-1 and HUVEC-C cells were seeded into 24-well plates at a concentration of 1×10^4 cells per well in a 0.5-ml volume. Both cell types were allowed to attach and multiply in M5 medium supplemented with 50 μg/ml ECGS and 100 μg/ml heparin for 24 hr prior to the experiment. As previously described, the HUVEC-C plates required the additional step of coating with 0.2% gelatin for attachment. The stock experimental solution was composed of a 10,000 μ*M* solution of 2-$MeOE_2$ solubilized in 100% DMSO stored at room temperature. The stock solution was serially (1:10) diluted with 100% DMSO resulting in each working concentration of 2-$MeOE_2$. The working solutions of 2-$MeOE_2$ (in 100% DMSO) were diluted (1:100) at the time of experimentation with fresh M5 medium yielding final concentrations of 0.01 μ*M*, 0.1 μ*M*, 1.0 μ*M*, 10.0 μ*M* and 100.0 μ*M* 2-$MeOE_2$, which were added directly to the culture plate wells at the beginning of the experiment. The control wells received similarly diluted DMSO (carrier) in M5 medium. Both the control and experimental wells had a resulting final concentration of 1.0% DMSO. The medium was decanted from all the wells and replaced daily with their respective identical milieu for 3 days. On the fourth day, cells were routinely released from the substratum with trypsin, and the trypsin was inactivated by the addition of an equal amount of M5 medium with 10% FBS. The cells were counted using a hemacytometer and expressed as number of cells per well. The counts from the experimental wells were normalized against the control well counts, and data were expressed as a percentage of the control (mean ± SEM).

Cell Cycle Analysis. RSE-1 and HUVEC-C cells were seeded into 24-well plates at a concentration of $1–5 \times 10^4$ cells per well as previously described. Both cell types were allowed to attach and multiply in M5 medium with supplemented 50 μg/ml ECGS and 100 μg/ml heparin for 24 hr. The solutions were prepared as previously described. The concentration 10.0 μ*M* 2-$MeOE_2$ was chosen as optimal based on previously determined cell count–derived dose-response curves. The spent medium was decanted, and fresh solutions were added daily for 2 days as previously described. The cells were tested at 24 and 48 hr after the first addition of 2-$MeOE_2$. At these time periods, the control and experimental wells were routinely trypsin released from the substrate, and the trypsin was inactivated by the addition of an equal amount of M5 medium with 10% FBS. The cells were removed from the tissue culture well and centrifuged. The supernatant was decanted, and the cells were resuspended in 1% NP40, 0.1% sodium citrate, 10 μg/ml propidium iodide in PBS to remove plasma membrane components, exposing the nuclei and fluorescently labeling the DNA. Cells were analyzed on a custom-built flow cytometer where the emitted fluorescence intensity maxima (520 nm) from ~10,000 cells post 488 nm excitation was collected in histogram form and later evaluated for percentage $G_{1/0}$, S, and G_2M using ModFit software.

Statistical Analyses. The data obtained in the studies of dose-response curves and cell cycle analysis were analyzed for statistical significance using the Student's *t* test.

Results

To test the general effects of 2-$MeOE_2$ on endothelium, the growth inhibition of differing concentrations of 2-$MeOE_2$ was measured against proliferating untreated cells of the same type over a period of 4 days (Fig. 1). The normal, proliferating endothelial cell line (HUVEC-C) had

an ED_{50} ~3.5 μM whereas the immortal endothelial cell line (RSE-1) had a similar ED_{50} ~2.2 μM. RSE-1 showed a greater effect at 2.2 μM 2-$MeOE_2$ with ~50% inhibition of treated cell growth compared to HUVEC-C cells at 3.5 μM 2-$MeOE_2$, which showed only ~27% inhibition of cell growth. Of note, the immortal RSE-1 cells were more sensitive and exhibited an effect at the low concentrations of 0.01–1.0 μM 2-$MeOE_2$ unlike HUVEC-C that showed no significant effect at these low concentrations. The 2-$MeOE_2$ dose-response curves demonstrated the lowest, active concentration (i.e., the lowest tested concentration at which the maximal effect was observed), to be 10.0 μM 2-$MeOE_2$ in both HUVEC-C and RSE-1 cell lines. HUVEC-C treated with 10.0 μM 2-$MeOE_2$ for 3 days showed inhibition of cell proliferation to 44.00% ± 5.72 (mean ± S.E.) of control ($P < 0.0001$). The same 2-$MeOE_2$ concentration produced an even more dramatic inhibition of cell proliferation in RSE-1 to only 7.87% ± 1.06 of control ($P < 0.0001$).

Figure 2 shows photomicrographs of the 2-$MeOE_2$-treated RSE-1 cells exhibiting the characteristic morphologic alterations after treatment with 10.0 μM 2-$MeOE_2$. Under inverted light microscopy, not only was a marked decrease in cell numbers displayed but also disruption of the monolayer, granular cytoplasm, and roughened cell margins. Similar changes were found in HUVEC-C cells treated with effective doses of 2-$MeOE_2$ (not shown).

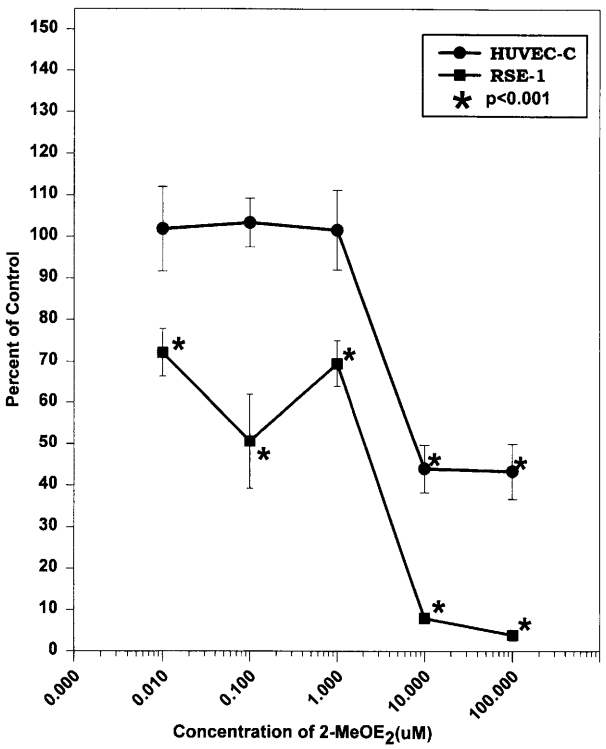

Figure 1. Dose-response curves. RSE-1 and HUVEC-C cells were seeded into 24-well plates at a concentration of 1 x 10^4 cells/well. Both cell types were allowed to attach and multiply in M5 medium supplemented with 50 µg/ml ECGS and 100 µg/ml heparin for 24 hr prior to the experiment. The working solutions of 2-$MeOE_2$ (in 100% DMSO) were diluted (1:100) at the time of experimentation with fresh M5 medium yielding final concentrations of 0.01 μM, 0.1 μM, 1.0 μM, 10.0 μM, and 100.0 μM 2-$MeOE_2$, which were added directly to the culture plate wells at the time of experimental onset. The control wells received similarly diluted DMSO (carrier) in M5 medium. Both the control and experimental wells had a resulting final concentration of 1.0% DMSO. The medium was replaced daily with their respective concentrations for 3 days. On the fourth day, cells were routinely released and counted using a hemacytometer and expressed as number of cells per well. The counts from the experimental wells were normalized using the control well counts and data expressed as a percentage of the control (mean ± SEM) ($n = 8$ for all data points).

After demonstrating the growth inhibitory effects of 2-$MeOE_2$ on these two types of endothelium, we performed flow cytometry to determine whether the growth inhibition resulted from a cell cycle–specific block. For both cell lines, we chose 10.0 μM 2-$MeOE_2$ to treat the experimental cells because it was the lowest, active concentration as previously described. Figure 3 shows representative histograms of the data generated by flow cytometry. In both HUVEC-C and RSE-1 cells treated with 10.0 μM 2-$MeOE_2$, there is a decrease in the $G_{1/0}$ peak with an increase in the G_2M peak of the cell cycle when compared with DMSO controls. Table I displays the percentages of these cells in the different compartments of the cell cycle. Both HUVEC-C and RSE-1 cells treated with 2-$MeOE_2$ demonstrated a decrease in the cells belonging to the G_2M and G_0 stage of the cell cycle. At 10.0 μM 2-$MeOE_2$, flow cytometric analysis of cell cycle

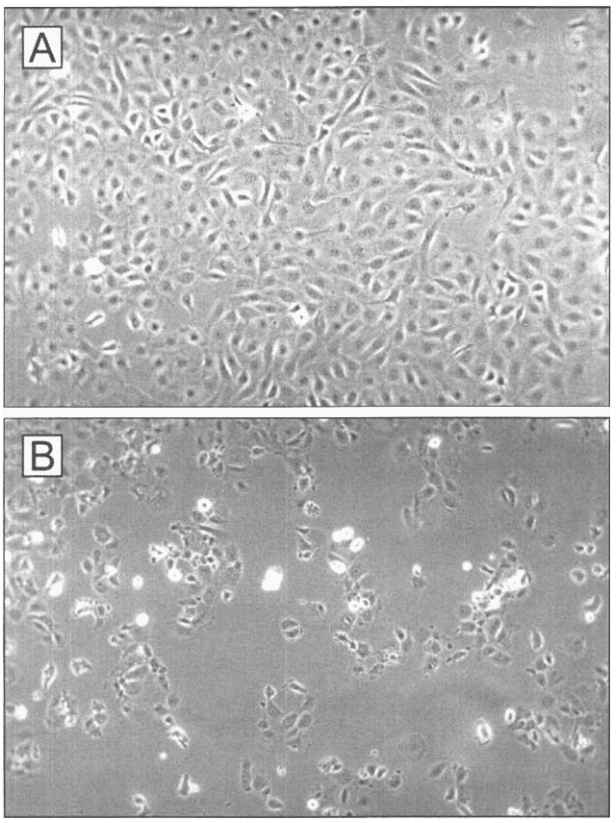

Figure 2. (A) A photograph of inverted light microscopy of 1 x 10^4 RSE-1 control cells treated with 1.0% DMSO for 24 hr exhibiting the characteristic endothelial cell morphology and a confluent monolayer (original magnification 25x). (B) A photograph of inverted light microscopy of 1 x 10^4 RSE-1 cells treated with 10.0 μM 2-$MeOE_2$ for 24 hr. Note the decreased cell numbers and an abnormal cell morphology with granular cytoplasm and roughened cell margins (original magnification 25x).

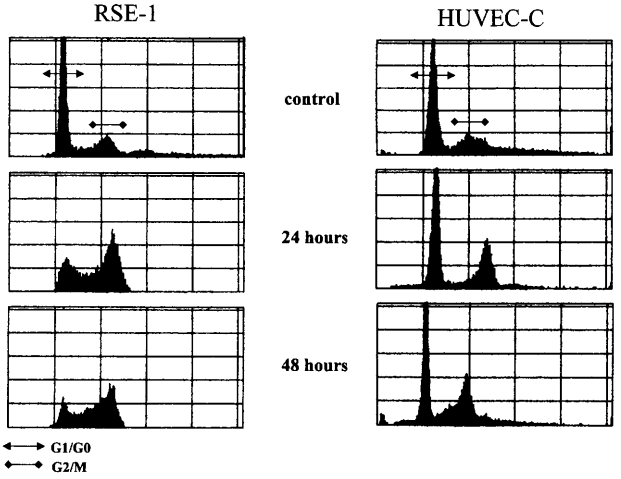

Figure 3. Cell cycle histograms. RSE-1 and HUVEC-C cells were seeded into 24-well plates at a concentration of 1–5 x 10^4 cells/well. Both cell types were allowed to attach and multiply in M5 medium supplemented with 50 µg/ml ECGS and 100 µg/ml heparin for 24 hr prior to the experiment. The working solutions of 2-$MeOE_2$ (in 100% DMSO) were diluted (1:100) at the time of experimentation with fresh M5 medium yielding final concentrations 10.0 µ*M* 2-$MeOE_2$, which was added directly to the culture plate wells at the time of experimental onset. The control wells received similarly diluted DMSO (carrier) in M5 medium. Both the control and experimental wells had a resulting final concentration of 1.0% DMSO. The medium was replaced daily with their respective concentrations for 2 days. At 24 and 48 hr, cells were routinely released, nuclear membranes exposed, DNA stained with propidium iodide, and cell cycle compartments determined by flow cytometry. The left peak represents the number of cells in G_1 and G_0. The right peak represents the cells in G_2 and M. The height of the peak determines the number of cells in these phases of the cell cycle. Both RSE-1 and HUVEC-C exposed to 2-$MeOE_2$ showed an increase in number of cells in G_2 and M with a concomitant decrease in G_1 and G_0.

events showed a cell cycle–specific block at G_2M in both HUVEC-C and RSE-1. After 48 hr of 2-$MeOE_2$-treatment, HUVEC-C cells in G_2M showed an increase to 31.73% ± 1.97 G_2M of the total population compared with the control 8.76% ± 2.08 G_2M ($P < 0.0001$). In RSE-1 the overall changes in the distribution of cells in the different phases of the cell cycle were similar but more pronounced. The response to 48 hr of treatment with 2-$MeOE_2$ was an increase to 36.26% ± 3.36% G_2M compared with control value of 18.84% ± 3.77 G_2M ($P < 0.0001$). RSE-1 cells in S-phase also increased from 28.97% ± 3.38 in control, to 54.3% ± 2.41 after treatment for 24 hr with 2-$MeOE_2$. This value remained similar at the 48-hr time period, suggesting that the block in RSE-1 cells also occurs at the S-phase. Conversely HUVEC-C cells showed no increase in S-phase at 24 hr with a control value of 25.32% ± 1.50% and an experimental value of 24.39% ± 2.44. At 48 hr, this value in fact decreased proportionally to the increase in G_2M, suggesting that the cells were still moving through S-phase and proceeding to the block in G_2M.

Discussion

2-$MeOE_2$ is a catecholestrogen endogenously produced in the human body, primarily generated by the peripheral conversion of estradiol(E_2) (6). 2-$MeOE_2$ circulates in the blood at a range of 30 p*M* in males to 30 n*M* in pregnant females, levels much lower than that at which its antiproliferative, antiangiogenic effects occur (7). However, serum levels do not necessarily reflect the intracellular level of 2-$MeOE_2$, which remains unknown and which may be higher due to the intracellular conversion of E_2.

E_2 is an inducer of mitosis and cell proliferation at physiologic concentrations in the body. 2-Hydroxyestradiol (2-OHE_2) is a major inducer of mitosis from intracellular conversion of E_2 by an estradiol 2-hydroxylase, whereas inhibition of this enzyme blocks the mitotic effect of estradiol (8). Of interest, at higher concentrations of 2-OHE_2 an antiproliferative effect occurs with the formation of an abnormal mitotic apparatus (8–9). Seegers *et al.* relates this

Table I. Effects of 2-$MeOE_2$ on Cell Cycle Components

Cell Type/Treatment	Cell Cycle Compartments (x ± SEM)					
	24 Hours			48 Hours		
	$\%G_{1/0}$	%S	$\%G_2M$	$\%G_{1/0}$	%S	$\%G_2M$
HUVEC-C ($n = 8$)	65.74 ± 1.39	25.32 ± 1.50	8.93 ± 0.78	68.05 ± 1.98	23.19 ± 3.64	8.76 ± 2.08
HUVEC-C + 10 µ*M* 2-$MeOE_2$ ($n = 7$)	55.46 ± 2.75[a]	24.39 ± 2.44[a]	20.15 ± 1.58[a]	52.67 ± 1.94[a]	15.61 ± 1.00[a]	31.73 ± 1.97[a]
RSE-1 ($n = 8$)	51.73 ± 5.76	28.97 ± 3.38	18.75 ± 2.26	60.15 ± 6.36	21.02 ± 2.71	18.84 ± 3.77
RSE-1 + 10 µ*M* 2-$MeOE_2$ ($n = 7$)	9.29 ± 0.59[a]	54.35 ± 2.41[a]	36.36 ± 2.37[a]	7.38 ± 0.95[a]	56.36 ± 3.41[a]	36.26 ± 3.36[a]

Note. RSE-1 and HUVEC-C cells were seeded into 24-well plates at a concentration of 1–5 x 10^4 cells/well. Both cell types were allowed to attach and multiply in M5 medium supplemented with 50 µg/ml ECGS and 100 µg/ml heparin for 24 hr prior to the experiment. The working solutions of 2-$MeOE_2$ (in 100% DMSO) were diluted (1:100) at the time of experimentation with fresh M5 medium yielding final concentrations 10.0 µ*M* 2-$MeOE_2$, which was added directly to the culture plate wells at the time of experimental onset. The control wells received similarly diluted DMSO (carrier) in M5 medium. Both the control and experimental wells had a resulting final concentration of 1.0% DMSO. The medium was replaced daily with their respective concentrations for 2 days. At 24 and 48 hr, cells were routinely released, nuclear membranes exposed, DNA stained with propidium iodide, and cell cycle compartments determined by flow cytometry. The table lists the percentages of cells in the different phases of the cell cycle for both control and experimental cells (10 µ*M* 2-$MeOE_2$) at time periods of 24 and 48 hr.
[a] $P < 0.0001$.

response to the formation of 2-$MeOE_2$ by intracellular catechol-o-methyltransferase (COMT) O-methylation of 2-OHE_2 because inhibition of O-methylation of 2-OHE_2 allows mitosis to continue normally (8). Therefore, a strong proliferative signal is sent to a cell by relatively large amounts of E_2-circulating in the serum and its intracellular conversion to 2-OHE_2. Perhaps the impact of this signal on cell proliferation is balanced intracellularly by the conversion of 2-OHE_2 to 2-$MeOE_2$.

2-$MeOE_2$ demonstrates a strong antiproliferative effect in this and other studies in a variety of cell types *in vitro* and *in vivo*. This mechanism of inhibition is not mediated through the estrogen receptor as 2-$MeOE_2$ exhibits little affinity for binding with cytosolic estrogen receptors (10). Furthermore, both estrogen receptor positive cells (MCF-7) and estrogen receptor negative cells (HeLa) are similarly inhibited (8). 2-$MeOE_2$ is probably a general growth inhibitor as it binds to the same site as colchicine on the microtubules, where it retards tubulin polymerization thereby altering microtubule structure and stability (7). Although 2-$MeOE_2$ is not as potent as colchicine and other synthetic compounds, it is the first potent, endogenously produced compound known to vigorously interact like colchicine at the assembly site of the spindle fiber (7). Attalla *et al.* found that physiologic low concentrations caused a mitotic block chiefly through kinetic stabilization of microtubules and alteration of microtubule spindle dynamics involving calmodulin rather than through inhibition of tubulin polymerization, which required higher concentrations of 2-$MeOE_2$ (11). 2-$MeOE_2$'s interference with microtubules probably blocks the ability of cells to proceed through the growth cycle and other cytosolic functions, because microtubules mediate multiple steps in cell division processes, morphology, cell migration, and cellular transport. Lottering *et al.* has suggested that 2-$MeOE_2$ inhibits protein phosphorylation, which prevents polymerization of microtubules and impairs the activation of $p34^{cdc2}$ crucial to the onset of mitosis (12). 2-$MeOE_2$ also interferes with PCNA function in DNA repair and induces p53 gene byproduct which propels rapidly dividing cells into apoptosis (12–13).

Our results suggest that the natural mammalian estrogen metabolite, 2-$MeOE_2$ inhibits the proliferation of human blood vascular endothelium (HUVEC-C) and angiotumor cells (RSE-1) *in vitro*. 2-$MeOE_2$ demonstrates a similar but more intense inhibitory effect on an angiotumor cell line compared with untransformed endothelium. This augmented effect is likely due to the faster doubling time of an immortal cell line compared with a normal cell line. Flow cytometric measurements of DNA-staining propidium iodide favors that 2-$MeOE_2$'s mechanism for growth inhibition is mediated through interference with microtubules and results in the specific blockage of cell cycle progression at G_2M and perhaps S-phase in immortal cells, rendering these cells unable to complete mitosis. Attalla *et al.* has suggested that this block occurs in anaphase due to the accumulation of cyclin B and cdc2, and the effect is reversible (11). We also documented a change in cell morphology of both HUVEC-C and RSE-1. This alteration is presumably due to altered microtubule function, and microtubules are structures responsible for maintaining cell shape.

Our results complement other studies that have suggested a role for 2-$MeOE_2$ as a new antiangiogenic, antiendothelial agent. *In vivo,* 2-$MeOE_2$ counteracts the cytokine-induced (b-FGF and VEGF) endothelial proliferative response whereas neoplasms manifest a notable reduction in tumor microvessel density (14–15). Some studies have also shown that 2-$MeOE_2$ blocks proliferation of normal and immortal cells, which are not endothelial in origin (8, 15–16). In this regard, 2-$MeOE_2$ acts as a nonspecific antiproliferative agent similar to other chemotherapeutic agents. Future studies need to examine 2-$MeOE_2$'s specific antiangiogenic effects as compared with its general inhibitory action against rapidly dividing cells.

In vivo, the antiproliferative effect of 2-$MeOE_2$ on tumor cells derives potentially from two separate processes. The first is a tumor cell–directed cytotoxicity. The second is 2-$MeOE_2$'s inhibitory effect for new blood vessel formation needed by the tumor for continued growth and metabolism. These *in vivo* effects occur without apparent toxicity to normal, nonproliferating cells (14–15).

Folkman and Ingber outlined three strategies for clinical application of angiogenic agents including 1) blocking the production and release of angiogenic molecules from tumor cells; 2) neutralizing secreted angiogenic molecules; and 3) inhibiting the response of vascular endothelial cells to angiogenic stimulation (3). 2-$MeOE_2$ inhibits the proliferative response of endothelial cells to angiogenic stimulation and also arrests the uncontrolled growth of immortal, angiotumor cells. Colchicine and similar agents that act at a specific tubulin site reduce cytoskeletal organization for cell movement (2). Fotsis *et al.* have also demonstrated 2-$MeOE_2$'s inhibition of endothelial cell migration and the ability to form capillary tubes in a collagen medium (14).

Angiogenesis is crucial to tumor growth, and antiangiogenic agents provide a new therapeutic intervention for cancer control, possibly in conjunction with conventional chemotherapy and also as initial therapeutic blockers against metastasis (3). Greater understanding of angiogenesis and its inhibition is also likely to shed light on the vascular pathophysiology of a variety of normal and other pathologic processes such as ischemia, wound healing, and vasculopathies. Newer agents with limited toxicity such as 2-methoxyestradiol have the potential to modulate hemangiogenesis and lymphangiogenesis for improved therapeutic benefit.

1. Folkman Judah. The role of angiogenesis in tumor growth. Cancer Biology **3**:65–71, 1992.
2. Auerbach W, Auerbach R. Angiogenesis inhibition: A review. Pharmacy Therapeutics **63**:265–311, 1994.
3. Folkman J, Ingber D. Inhibition of angiogenesis. Cancer Biology **3**:89–96, 1992.

4. Folkman J. Clinical applications of research on angiogenesis. Seminars in Medicine of the Beth Israel Hospital, Boston **333**(26):1757–1763, 1993.
5. Way D, Borgs P, Bernas M, Ramirez G, Shao X, Witte M, Weinand M, Riedel U, Johnson V, Witte C. Characterization of an established immortal endothelial cell line (RSE-1): Comparison to AIDS-Kaposi sarcoma cell cultures. Lymphology **27**(Suppl):761–762, 1993.
6. Breuer H, Knuppen R. Biogenese von 2-methoxy-oestradiol in der menschlichen Leber. Natur Wissenchaften **12:**47–48, 1960.
7. D'Amato R, Lin C, Flynn E, Folkman J, Hamel E. 2-Methoxyestradiol, an endogenous mammalian metaboite, inhibits tubulin polymerization by interacting at the colchicine site. Proc Natl Acad Sci U S A **91:**3964–3968, 1994.
8. Seegers J, Aveling M, van Aswegen C, Marrisa C, Koch F, Joubert, W. The cytotoxic effects of estradiol-17β, catecholestradiols and methoxyestradiols on dividing MCF-7 and HeLa Cells. J Steroid Biochem Mol Biol **32**(6):797–809, 1989.
9. Nishigaki I, Sasaguri Y, Yagi K. Anti-proliferative effect of 2-methoxyestradiol on cultured smooth muscle cells from rabbit aorta. Atherosclerosis **133:**167–170, 1995.
10. Cushmann M, Hu-Ming H, Katzenellenbogen Lin C, Hamel E. Synthesis, antitubulin and antimitotic activity, and cytotoxicity of analogs of 2-methoxyestradiol, an endogenous mammalian metabolite of estradiol that inhibits tubulin polymerization by binding to the colchicine binding site. J Med Chem **38**(12):2041–2049, 1995.
11. Attalla H, Makela T, Adelercreutz H, Andersson L. 2-methoxyestradiol arrests cells in mitosis without depolymerizing tubulin. Biochem Biophys Res Commun **228:**467–473, 1996.
12. Lottering M, de Kock M, Theorickus V, Grobler C, Seegers J. 17β-estradiol metabolites affect some regulators of the MCF-7 cell cycle. Cancer **110:**181–186, 1996.
13. Mukhopadhyay T, Roth J. Induction of apoptosis in human lung cancer cells after wild-type p53 activation by methoxyestradiol. Oncogene **14:**379–384, 1997.
14. Fotsis T, Zhang Y, Pepper M, Adlercreutz H, Montesano R, Nawroth P, Schweigerer L. The endogenous oestrogen metabolite 2-methoxyestradiol inhibits angiogenesis and suppresses tumor growth. Nature **368:**237–239, 1994.
15. Klauber N, Parangi S, Flynn E, Hamel E, D'Amato R. Inhibition of angiogenesis and breast cancer in mice by the microtubule inhibitors 2-methoxyestradiol and taxol. Cancer Res **57:**81–86, 1997.
16. Spicer L, Hammond J. Catecholestrogens inhibit proliferation and DNA synthesis of porcine granulosa cells *in vitro:* Comparison with estradiol, 5α-dihydrotestosterone, gonadotropins, and catecholamines. Mol Cell Endocrinol **64:**119–126, 1989.