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Inhibition of Angiogenesis and Breast Cancer in Mice by the Microtubule Inhibitors 2-Methoxyestradiol and Taxol[1]

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ABSTRACT

2-Methoxyestradiol (2-ME), an endogenous estrogen metabolite which disrupts microtubule function, has been shown to inhibit proliferating cells *in vitro* and suppress certain murine tumors *in vivo*. *In vitro* screening has determined that breast cancer cell lines are most sensitive to inhibition by 2-ME. Additionally, 2-ME has been shown to inhibit angiogenesis *in vitro*. We tested whether 2-ME suppresses cytokine-induced angiogenesis *in vivo* and inhibits growth of a human breast carcinoma in severe combined immunodeficient mice. A model of basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF)-induced corneal neovascularization in C57BL/6 mice was used to evaluate the antiangiogenic effects of 2-ME and other microtubule inhibitors such as Taxol, vincristine, and colchicine. 2-ME (150 mg/kg p.o., $n = 20$) inhibited bFGF and VEGF-induced neovascularization by 39% and 54%, respectively. Taxol (6 mg/kg i.p., $n = 17$) inhibited bFGF and VEGF-induced neovascularization by 45% and 37%, respectively. Vincristine (0.2 mg/kg i.p., $n = 8$) and colchicine (0.25 mg/kg i.p., $n = 8$) had no effect. Treatment with 2-ME (75 mg/kg p.o., $n = 9$) for 1 month suppressed the growth of a human breast carcinoma in mice by 60% without toxicity. Recognition of the antiangiogenic and antitumor properties of 2-ME and Taxol may be crucial in planning clinical applications to angiogenesis-dependent diseases.

INTRODUCTION

2-ME[3] is a naturally occurring mammalian metabolite of estradiol (1). Recent interest in 2-ME has resulted from its ability to inhibit proliferating cancer cells (2–4) and angiogenesis *in vitro* (2, 5). The mechanism of 2-ME effects on cellular proliferation has been attributed to its ability to bind to the colchicine-binding site of tubulin, resulting in inhibition of tubulin polymerization or formation of polymer with altered stability properties and morphology, depending on the reaction conditions (5, 6).

Angiogenesis is required for the growth of solid tumors (7–9). 2-ME inhibited endothelial cell proliferation, migration, and tube formation *in vitro*, and, when given p.o. to mice, inhibited the growth of certain murine solid tumors with an accompanying decrease in their vessel density (2). Because 2-ME is cytotoxic to tumor cells, it may indirectly inhibit tumor angiogenesis (2) by killing tumor cells that produce the angiogenic growth factors. Whether 2-ME directly inhibits cytokine-induced angiogenesis in the absence of tumor cytotoxicity *in vivo* has not yet been elucidated. Therefore, we examined the effect of p.o. administered 2-ME in a mouse corneal neovascularization model induced by the potent angiogenesis stimulator bFGF. Additionally, to determine whether microtubule inhibitors in general are capable of inhibiting angiogenesis (or whether this is a unique feature), we compared the antiangiogenic activity of 2-ME to other known microtubule inhibitors such as paclitaxel (Taxol), vincristine, and colchicine.

Microtubule inhibitors such as Taxol have been shown to be efficacious against breast cancer (10). Similarly, when 2-ME was examined in the National Cancer Institute Developmental Therapeutics Program's *in vitro* panel of approximately 55 human cancer cell lines, human breast cancer cells (both estrogen receptor positive and negative) were the most sensitive to the cytotoxic effects of 2-ME *in vitro* (11). Whether or not 2-ME suppresses breast cancer growth *in vivo* is unknown. Therefore, we tested daily p.o. administration of 2-ME on the growth of an estrogen receptor-negative human breast carcinoma for 1 month in mice. By analyzing the proliferation rate and microvessel density of resected tumors with immunohistochemistry, we were able to delineate direct tumor cytotoxicity and inhibition of angiogenesis.

MATERIALS AND METHODS

Cells and Culture Conditions. MDA-MB-435 human breast carcinoma cells were grown in RPMI 1640 containing 10% heat-inactivated FCS (Hyclone Laboratories, Logan, UT) and supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin (Irvine Scientific, Santa Ana, CA). BCE cells were obtained as described previously (12) and grown on gelatinized surfaces [1.5 g/100 ml of gelatin in PBS (0.2 g/liter KCl, 0.2 g/liter KH_2PO_4, 8 g/liter NaCl, and 1.15 g/liter Na_2HPO_4)] in DMEM containing 10% heat-inactivated bovine CS and supplemented with L-glutamine (2 mM), penicillin (110 units/ml), and streptomycin (100 μg/ml) and 3 ng/ml bFGF. Both cell types were incubated at 37°C under 10% CO_2 in air. RPMI 1640, DMEM, and CS were obtained from JRH Biosciences (Lenexa, KS).

Proliferation Assays. MDA-MB-435 cells were plated at 20,000 cells/ml in 24-well dishes. BCE cells were plated at 24,000 cells/ml into gelatinized 24-well dishes. After allowing the cells to attach overnight, the appropriate fresh media were applied containing differing concentrations of 2-ME. Drug was made soluble in DMSO (Fisher Scientific, Pittsburgh, PA), and control wells received equal volumes (0.1%) of vehicle alone. Drug was added to the wells in a volume of 500 μl. The media for BCE cells was supplemented with

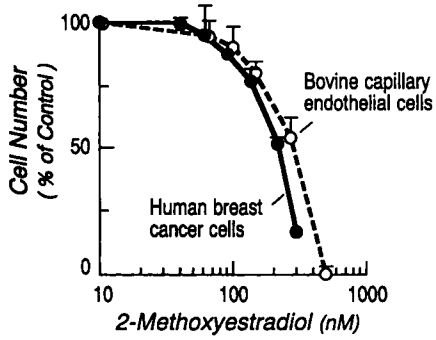

Fig. 1. Increasing concentrations of 2-ME were applied to BCE cells in the presence of 1 ng/ml bFGF (○) or MDA-MB-435 human breast carcinoma cells (●) as described in "Materials and Methods" in a 72-h proliferation assay. 2-ME inhibited the proliferation of both cell types in a concentration-dependent matter. Data are presented as means and experimental wells were repeated in triplicate. *Bars*, SE.

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[3] The abbreviations used are: 2-ME, 2-methoxyestradiol; bFGF, basic fibroblast growth factor; BCE, bovine capillary endothelial; SCID severe combined immunodeficient; CS, calf serum; VEGF, vascular endothelial growth factor; PCNA, proliferating cell nuclear antigen; CAM, chorioallantoic membrane.

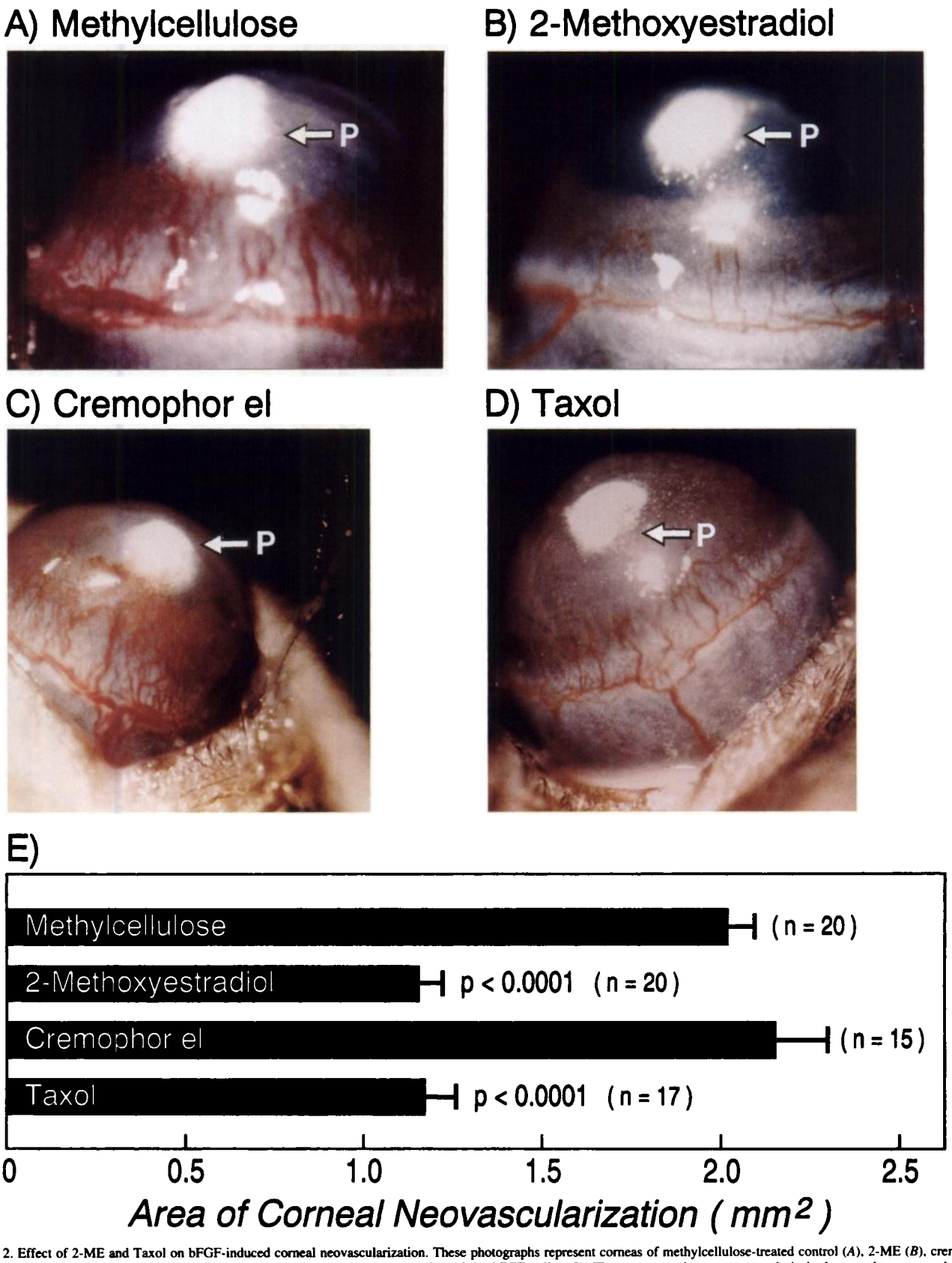


Fig. 2. Effect of 2-ME and Taxol on bFGF-induced corneal neovascularization. These photographs represent corneas of methylcellulose-treated control (*A*), 2-ME (*B*), cremophor el-treated control (*C*), or Taxol-treated C57BL/6 mice (*D*) 5 days after implantation of the bFGF pellet (*P*). There are prominent new vessels in both control corneas, whereas a suppression of the vascular response is seen after treatment with either 2-ME or Taxol. *E*, quantitation of the area of neovascularization of methylcellulose-treated control, 2-ME-treated, cremophor el-treated control, and Taxol-treated mice.

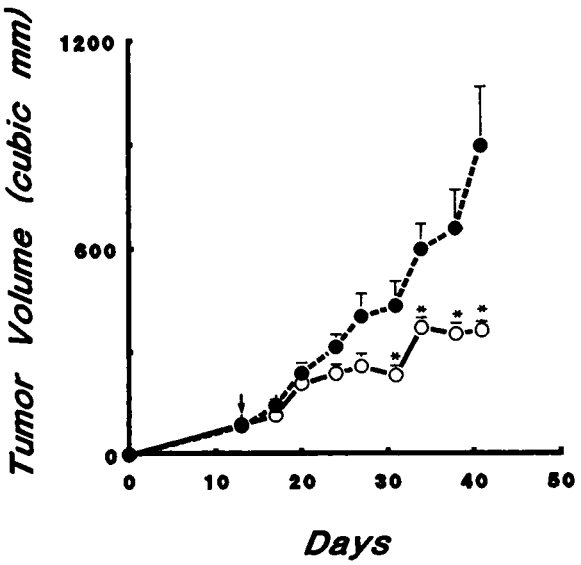


Fig. 3. Effect of 2-ME on the growth of an estrogen receptor-negative human breast carcinoma. SCID mice were inoculated with MDA-MB-435 on day 0, and treatment with either methylcellulose (●, $n = 11$) or 75 mg/kg 2-ME p.o. (○, $n = 9$) was started on day 12 after the tumors became measurable (*arrow*). Tumor protocol and measurement procedures are described in "Materials and Methods." 2-ME was significantly different (*, $P < 0.05$) from controls from day 31 to the end of the study. *Bars*, SE.

bFGF (1 ng/ml). BCE cells were assayed in 5% CS, whereas MDA-MB-435 were assayed in 2.5% FCS because of their more rapid growth curves. The cells were incubated for 3 days at 37°C and then washed with PBS, detached by trypsinization (0.05 g/100 ml trypsin, 0.53 mM EDTA, from Life Technologies, Inc., Grand Island, NY), resuspended in Hematall (Fisher Scientific), and counted using a Coulter Counter. Each condition was prepared in triplicate, and the experiments were carried out three times. Results are presented as means ± SE.

Mice. Five- to 7-week-old SCID female mice were obtained from Massachusetts General Hospital (Boston, MA). Immunocompetent 7–9-week-old female C57BL/6 mice were obtained from ARCH Technical Services, Children's Hospital (Boston, MA). All animal studies were conducted according to protocols approved by the Animal Ethics Committee of Children's Hospital. Animals were anesthetized in a methoxyflurane (Pittman-Moore, Mundelein, IL) chamber prior to all procedures and were observed until fully recovered. Animals were sacrificed by a lethal dose of methoxyflurane.

Mouse Corneal Micropocket Assay. To study the effect of microtubule inhibitors on angiogenesis *in vivo*, a model of angiogenesis in the mouse cornea was used in C57BL/6 mice as described previously (13). In brief, after animals were anesthetized, corneal micropockets were created in both eyes with a modified von Graefe cataract knife. Into each pocket, a 0.4 mm × 0.4 mm × 0.2-mm sucrose aluminum sulfate (Bukh Meditec, Copenhagen, Denmark) pellet coated with hydron polymer type NCC (IFN Sciences, New Brunswick, NJ) containing 80 ng of bFGF or 160 ng of human recombinant VEGF (gift from Reprogenesis, Cambridge, MA) was implanted 1.0–1.2 mm from the limbal vessels (bFGF experiments) or 0.5–0.7 mm from the limbus (VEGF experiments). Erythromycin ointment (E. Fougera, Melville, NY) was applied to each operated eye. The sucralfate acts to stabilize the growth factor and to slow its release from the hydron. Before testing each drug in this assay, we found the maximal dose that could be administered daily for 5 consecutive days without producing signs of toxicity (*i.e.*, no hair loss, diarrhea, infection, lethargy, or weight loss). The treated groups received daily administration for 5 consecutive days of 150 mg/kg (0.1 ml) of 2-ME suspended in 0.5 g/100 ml of carboxymethylcellulose p.o., 0.25 mg/kg (0.1 ml) colchicine suspended in 0.9 g/100 ml of NaCl solution i.p., 6 mg/kg (0.1 ml) Taxol (Calbiochem, San Diego, CA) suspended in cremophor el i.p., or 0.2 mg/kg (0.1 ml) i.p. vincristine sulfate injection, USP (Eli Lilly and Co., Indianapolis, IN). Colchicine and cremophor el were purchased from Sigma Chemical Co. (St. Louis, MO). Treatment was started on the day of pellet implantation. Control mice for the 2-ME-, colchicine-, and Taxol-treated mice received 0.1 ml of carboxymethylcellulose p.o., NaCl solution i.p., or cremophor el i.p., respectively. The vascular response (measured as the maximal vessel length and number of clock hours of neovascularization) was assessed on the fifth postoperative day, which was found to be the day of maximal angiogenic response. For this purpose, the eyes of the mice were examined by slit-lamp biomicroscopy. Area of corneal neovascularization was calculated using a modified formula for a half-ellipse: Area (mm^2)= [(π × clock hours × length (mm) × 0.2 mm]. This formula provides the most accurate approximation of the area of neovascularization that grows toward the pellet (13).

Tumor Growth *in Vivo*. To determine the antitumor activity of 2-ME, we s.c. inoculated SCID mice with 10^6 MDA-MB-435 estrogen receptor-negative human breast carcinoma cells. Treatment was initiated on day 12 when tumor volumes reached 75–100 mm^3 in volume. 2-ME (75 mg/kg) was suspended in 0.5 g/100 ml of carboxymethylcellulose with sterile glass beads, vortexed for 5 min, and administered p.o. in a volume of 0.1 ml. Control mice received 0.1 ml of carboxymethylcellulose p.o. Serial caliper measurements of perpendicular diameters were used to calculate tumor volume using the following formula: (shortest diameter)^2 × (longest diameter) × 0.52.

Immunohistochemistry. Tumor tissues were fixed in Carnoy's fixative overnight and embedded in paraffin according to standard histological procedures. Carnoy-fixed tissue sections (5–8 μm) were pretreated with 2 μg/ml proteinase K (Boehringer Mannheim, Mannheim, Germany) at 37°C for 15 min before staining with a rabbit polyclonal antibody against human von Willebrand factor (DAKO, Carpinteria, CA). Positive staining was detected by incubating sequentially with a secondary antibody against rabbit conjugated to horseradish peroxidase (DAKO) and diaminobenzidine tetrahydrochloride (DAKO) as a chromagen. Sections were counterstained with methyl green (Schmid & Co., Stuttgart, Germany) and mounted in Permount (Fisher Scientific, Fair Lawn, NJ). Microvessel density was determined by light microscopy according to the procedure of Weidner *et al.* (14). Each count was expressed as the number of microvessels identified within a selected ×250 field. At least three separate ×250 fields were analyzed for each tumor specimen.

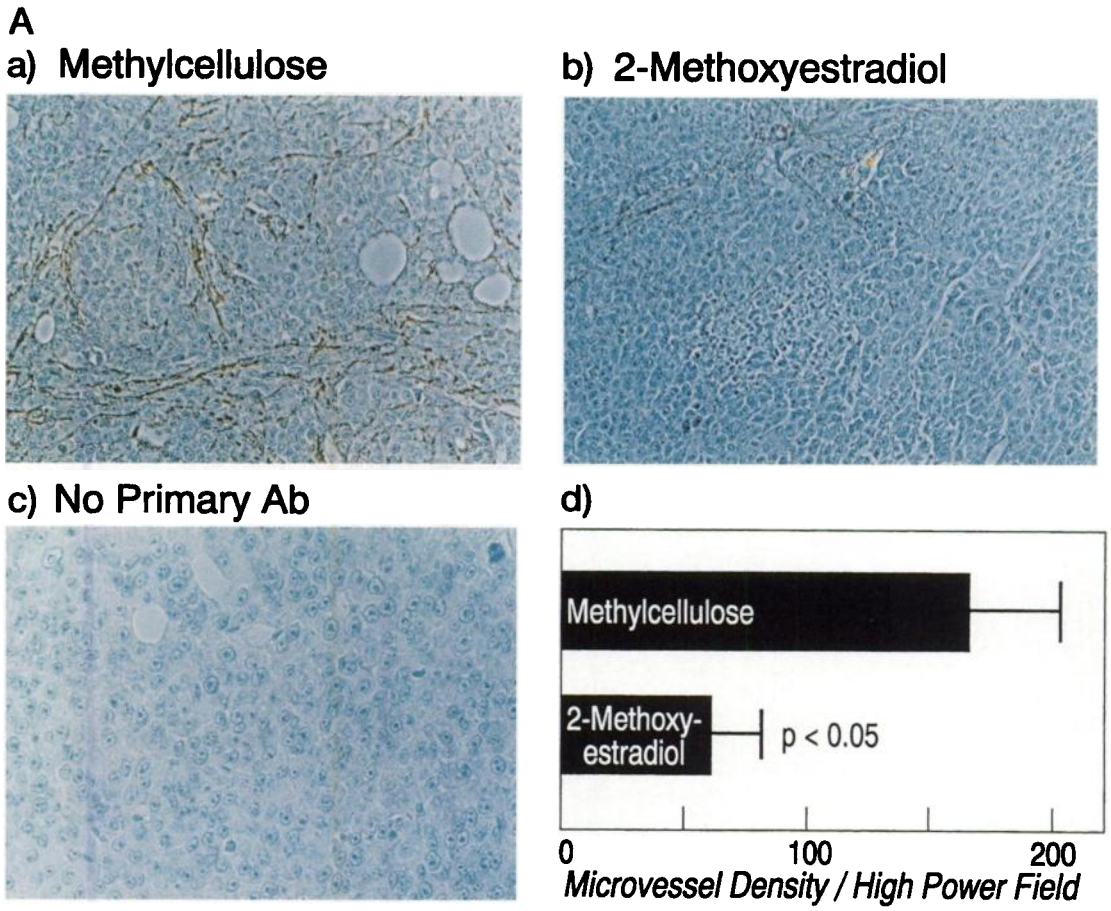
For PCNA staining, Carnoy-fixed sections (5–8 μm) were pretreated in target unmasking fluid (Signet Laboratories, Dedham, MA) for 10 min at 90°C for antigen retrieval. Immunohistochemical staining was performed with an anti-PCNA murine monoclonal antibody (Signet). Positive staining was detected by using horseradish peroxidase-conjugated secondary (DAKO) and tertiary (DAKO) antibodies and diaminobenzidine tetrahydrochloride as a chromagen. The PCNA-labeling index was determined by counting the percentage of stained cells under light microscopy within selected ×630 fields. A minimum of 2000 cells were counted for each tumor specimen.

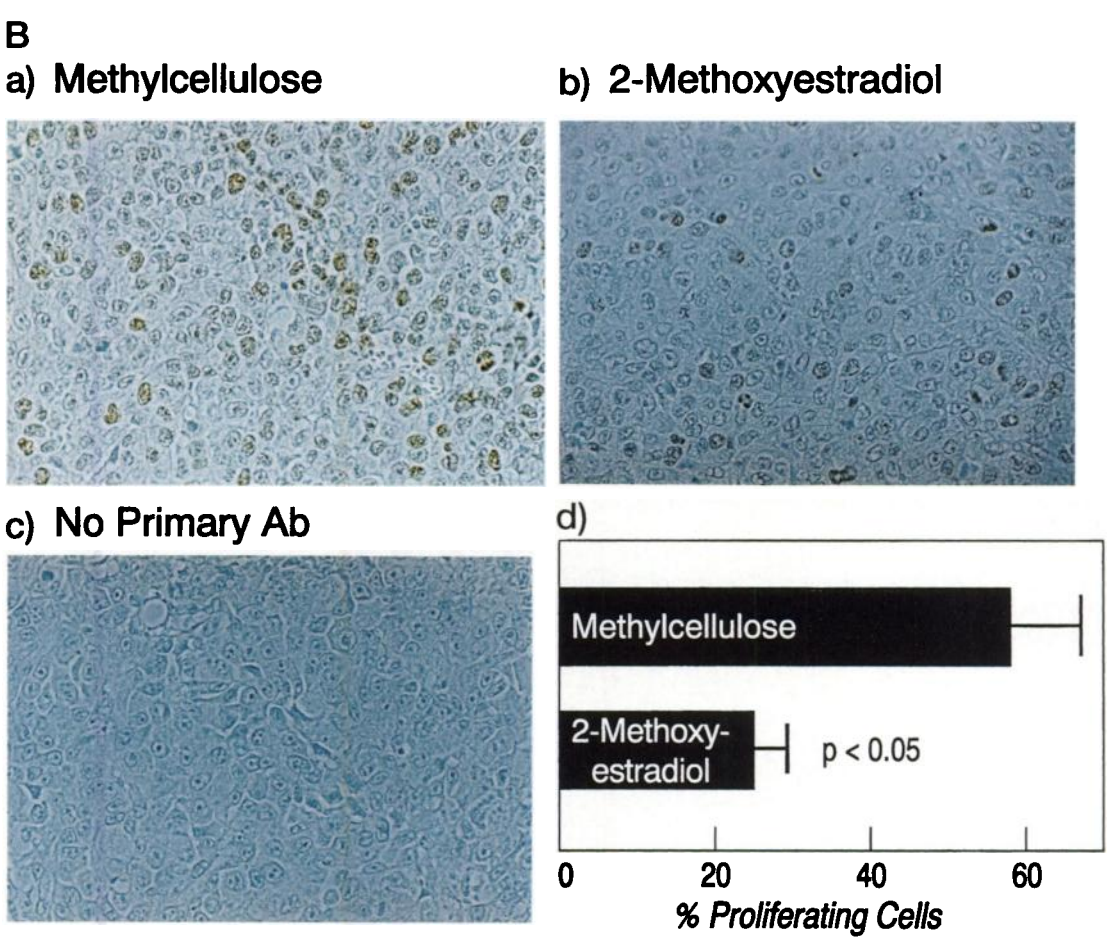
Statistical Analysis. Student's two-tailed *t* test was used to calculate the statistical significance of differences between groups for all experiments. The results are presented as means ± SE.

RESULTS

Proliferation Assays. 2-ME inhibited the proliferation of both BCE cells and MDA-MB-435 cells in a concentration-dependent manner (Fig. 1). The half-maximal inhibitory concentration for BCE cells was 191 ± 13 nM and for MDA-MB-435 cells was 195 ± 5 nM.

Mouse Corneal Micropocket Assay. For each drug tested, we used the maximally tolerated dose that we found could be administered for 5 days without signs of toxicity or weight loss. Treatment of C57BL/6 mice with 150 mg/kg 2-ME p.o. inhibited the area of bFGF-induced corneal neovascularization by 39% ($n = 20$ control methylcellulose, $n = 20$ for 2-ME, $P < 0.0001$; Fig. 2) in two experiments and inhibited the area of VEGF-induced corneal neovascularization by 54% ($n = 6$ control, $n = 7$ for 2-ME, $P < 0.005$). Treatment of mice with 6 mg/kg Taxol i.p. inhibited the area of bFGF-induced corneal neovascularization by 45% ($n = 15$ control cremophor el, $n = 17$ for Taxol, $P < 0.0001$) in two experiments (Fig. 2) and inhibited the area of VEGF-induced corneal neovascularization by 37% ($n = 6$ control, $n = 6$ for Taxol, $P < 0.05$). Treatment with 0.25 mg/kg colchicine i.p. had no effect on bFGF-induced corneal neovascularization ($n = 8$ control saline, $n = 8$ for colchicine) or VEGF-induced corneal neovascularization ($n = 6$ control, $n = 7$ for colchicine). Similarly, treatment with 0.2 mg/kg vincristine i.p. had no





effect on the area of bFGF-induced corneal angiogenesis (n = 8 control saline, n = 8 for vincristine) or VEGF-induced corneal angiogenesis (n = 6 control, n = 7 for vincristine). Weight loss >10% occurred when Taxol, colchicine, and vincristine were administered at higher doses (Taxol, 12 mg/kg); colchicine, 0.5 mg/kg; and vincristine, 0.5 mg/kg).

Tumor Growth *in Vivo*. Treatment with 2-ME (n = 9) at 75 mg/kg p.o. daily for 29 days was effective at suppressing the growth of a MDA-MB-435 human breast carcinoma in SCID mice as compared with control (n = 11) methylcellulose-treated mice. The groups were significantly different ($P < 0.05$) from day 31 through the end of the study on day 42, with a 60% suppression of tumor volume (Fig. 3). The dose of 75 mg/kg was chosen because this was the maximal dose that produced no signs of toxicity (*i.e.*, no hair loss, diarrhea, infection, lethargy, or weight loss) after 29 days of treatment. Treatment at a higher dose (150 mg/kg p.o. daily, n = 8) produced 15% weight loss after 25 days of treatment.

To determine what angiogenic factors are produced by MDA-MB-435 cells (to correlate with the preceding micropocket studies), we demonstrated that both bFGF (14 pg/10^6 cells) and VEGF (2.3 ng/10^6 cells) are detectable in the supernatant of these cells when cultured for 48 h.

Immunohistochemistry. Since 2-ME inhibited tumor cell proliferation *in vitro*, we determined the proliferation rate of the MDA-MB-435 human breast carcinomas to see whether tumor proliferation was directly inhibited *in vivo*. Tumors were analyzed by immunohistochemical staining using antibodies to PCNA, which is expressed in late G_1 to M (15). There was a 57% decrease in the proliferation rate of tumor cells from 2-ME-treated mice (n = 3, $P < 0.05$) as compared to tumor cells from control methylcellulose-treated mice (n = 3, Fig. 4,*a*).

To determine the vascularity of the tumors, immunohistochemical staining with antibodies against von Willebrand factor (an endothelial cell-specific marker) was performed. There was a 60% inhibition of microvessel density in tumors from 2-ME-treated mice (n = 3, $P < 0.05$) as compared to tumors from control methylcellulose-treated mice (n = 3, Fig. 4, *b*).

DISCUSSION

Anticancer agents have previously been evaluated for their antiangiogenic potential on the chick CAM with the hypothesis that the destruction of solid tumors by antitumor agents is mediated in part by local loss of the vasculature (16). Epirubicin, doxorubicin, mitoxantrone, vinblastine, vincristine, and Taxol have been shown to have some antiangiogenic activity on the CAM (16–18). In contrast, cyclophosphamide, melphan, 5-fluorouracil, methotrexate, and *cis*-platinum were inactive (16). These experiments suggest that only a subset of antimitotics are potentially effective angiogenesis inhibitors. Furthermore, it is likely that only a few compounds from this subset of antimitotics which are active on the CAM will inhibit angiogenesis when given systemically *in vivo*. This is due to the fact that the local concentrations of a compound released by pellets placed on the surface of the CAM are far higher than the circulating concentrations obtainable in the blood when administered systemically to animals. Additionally, metabolic inactivation of compounds can occur when drugs are given systemically but not when pellets are placed topically on the CAM.

We examined the effect of 2-ME administered p.o. on bFGF- and VEGF-induced corneal neovascularization *in vivo* and compared its effect with other microtubule inhibitors administered systemically. Both 2-ME and Taxol significantly inhibited bFGF- and VEGF-induced corneal neovascularization, whereas vincristine and colchicine lacked antiangiogenic activity.

Vincristine and colchicine act by inhibiting microtubule polymerization, whereas Taxol causes formation of polymers with altered stability properties. The mechanism of action of 2-ME appears to be more complex. This compound binds to both unpolymerized tubulin at the colchicine site and to tubulin polymers at a less well-defined site. When large amounts of 2-ME bind to polymer, this polymer is more stable than polymer formed in the absence of drug (5, 6). Therefore, endothelial cells *in vivo* may be more susceptible to microtubule inhibitors that alter polymer stability than microtubule inhibitors that inhibit microtubule polymerization. Our results demonstrate that not all microtubules inhibitors are capable of inhibiting angiogenesis *in vivo*, confirming that only a subset of antimitotics are antiangiogenic. Our finding that 2-ME inhibits bFGF- and VEGF-induced corneal neovascularization *in vivo* corroborates previous findings that 2-ME inhibits angiogenesis *in vitro* (2, 5) and decreases tumor microvessel density *in vivo* (2).

Taxol has been reported to be particularly effective at inhibiting proliferation of breast cancer growth (10). Since 2-ME also affects microtubule polymer stability and was shown to be most cytotoxic to breast cancers *in vitro*, we tested p.o. administered 2-ME on the growth of a human breast carcinoma *in vivo*, which is estrogen receptor negative. Previous studies have shown that 2-ME does not bind to the estrogen receptor (19) and therefore we expect the actions of 2-ME on breast cancer to be estrogen receptor independent. This is supported by the National Cancer Institute Developmental Therapeutics Program's *in vitro* panel, where 2-ME potently inhibits the proliferation of both estrogen receptor-positive (MCF-7) and -negative cell lines. One month of daily p.o. treatment with 2-ME resulted in significant suppression of tumor growth, with no weight loss or apparent signs of toxicity. Immunohistochemical analysis demonstrates that there is a decrease in both the tumor vessel density and tumor cell proliferation rate, which is consistent with the finding that 2-ME inhibits both capillary endothelial cell and breast cancer cell proliferation *in vitro*. Thus, 2-ME appears to inhibit tumor growth through a combined direct inhibition of both the endothelial cell and tumor cell compartments.

Although Taxol is known to be effective at inhibiting tumor growth, long-term administration is limited due to toxicity. 2-ME, however, was well tolerated after 1 month of daily treatment in mice. Therefore, we suggest that 2-ME may serve as a chronic p.o. therapy following acute i.v. treatment of tumors with Taxol to prolong tumor suppression.

Fig. 4. *A*, immunohistochemical analysis of vascularization of human breast carcinomas from methylcellulose-treated control and 2-ME-treated mice (×250). Animals were sacrificed 42 days after tumor inoculation. Tumor vascularization was analyzed by staining with polyclonal antibodies against von Willebrand factor, an endothelial cell specific marker, as described in "Materials and Methods." *a*, in mice treated with methylcellulose, there was marked neovascularization (brown stain). *b*, in contrast, mice treated with 2-ME had a marked decrease in tumor microvessel density. *c*, methylcellulose-treated tumor with primary antibody omitted, showing no background staining. *d*, quantitation of microvessel density of methylcellulose (n = 3) and 2-ME-treated (n = 3) human breast carcinomas. *B*, immunohistochemical analysis of proliferation of human breast carcinomas from methylcellulose-treated control and 2-ME-treated mice (×400). Tumor proliferation was analyzed by staining with antibodies against PCNA as described in "Materials and Methods." *a*, in mice treated with methylcellulose, there was marked immunoperoxidase staining (brown stain). *b*, in contrast, mice treated with 2-ME had a decrease in immunoperoxidase staining. *c*, methylcellulose-treated tumor with the primary antibody omitted, showing no background staining. *d*, quantitation of proliferation rate of methylcellulose-treated (n = 3) and 2-ME-treated (n = 3) human breast carcinomas. *A*, ×250; *B*, ×400.

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