Novel 2-Methoxyestradiol Analogues with Antitumor Activity

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ABSTRACT

2-Methoxyestradiol (2-ME2) is a natural estrogen metabolite that, while devoid of estrogenic effects, has both antiangiogenic and antitumor effects. 2-ME2 is currently being evaluated in Phase I and Phase II clinical trials for the treatment of multiple types of cancer. Novel analogues of 2-ME2 were tested for activities that predict antiangiogenic and antitumor effects. Selected analogues were tested for inhibitory activity against endothelial cell proliferation and invasion. The results show that these analogues are effective inhibitors of endothelial cell activities that may predict antiangiogenic activity, and one analogue, 2-methoxy-14-dehydro-2-ME2, was 6–15-fold more potent than the parent compound in these assays. The analogues were also evaluated for inhibition of proliferation and cytotoxicity against multiple tumor cell lines and found to be potent and effective. 14-Dehydro-2-ME2 was approximately 15-fold more potent than 2-ME2 against various tumor cell lines, and 2-methoxy-15-dehydro-2-ME2 was particularly effective against DU 145 and PC3 prostate cancer cell lines. In vivo antitumor activity was observed for the three analogues tested in the murine xenograft MDA-MB-435 model; however, 2-ME2 provided no antitumor activity in this trial. The two most effective analogues, 14-dehydro-2-ME2 and 2-methoxy-15-dehydro-2-ME2, provided 29.4% and 26.7% inhibition of tumor burden, respectively. Mechanism of action studies indicate that the analogues cause mitotic spindle disruption, mitotic arrest, microtubule depolymerization, and inhibition of the assembly of purified tubulin similar to the effects of 2-ME2. Consistent with antimitotics that inhibit the dynamic instability of tubulin and initiate apoptosis, these novel 2-ME2 analogues cause Bcl-2 phosphorylation and activation of mitogen-activated protein kinase signaling pathways.

INTRODUCTION

The search for superior drugs for the treatment of cancer has focused recently on the process of angiogenesis. Judah Folkman proposed over 30 years ago that angiogenesis was critical for tumor development and that limiting the development of new blood vessels to a tumor could be a valuable target for anticancer therapy because without a new blood supply, tumors are limited to 1–2 mm in size (1). Angiogenesis is an attractive target for cancer chemotherapy because it provides an opportunity for more specific, less toxic drugs (2, 3).

The development of drug resistance in the genetically unstable tumor tissue is a cause of treatment failure. Targeting the genetically stable endothelial cells of the developing tumor holds great promise for more specific and less toxic treatment that will not become susceptible to multidrug resistance (4). Several new antiangiogenic agents are currently in the clinic, and data show that some of the more useful cancer chemotherapeutic drugs have antiangiogenic properties (5).

2-ME2 is a natural metabolite of estrogen that is devoid of estrogenic effects. The ability of this compound to target endothelial cells was first identified by its antiproliferative activity against microvascular and large vessel endothelial cells (6). Additional studies showed that 2-ME2 inhibits the migration and invasion of capillary endothelial cells and their ability to form capillary-like structures on collagen gels (6, 7). As predicted from the in vitro studies, 2-ME2 has antiangiogenic activity in several models (7, 8). Angiogenesis induced by basic fibroblast growth factor or vascular endothelial growth factor was inhibited by the oral administration of 2-ME2 in the murine corneal neovascularization assay (8). In addition to the antiangiogenic activity, 2-ME2 inhibits the proliferation of human tumor cells and initiates apoptosis in vitro (7, 9–11).

In murine models, 2-ME2 is an effective antitumor agent. The oral administration of 2-ME2 reduced tumor burden and neovascularization with little evidence of toxicity (6). In a murine xenograft model using MDA-MB-435 cells, 2-ME2 reduced tumor burden by 60% (8), and in a xenograft model with H460 non-small cell lung carcinoma, 2-ME2 inhibited tumor growth by 70% (12). 2-ME2 inhibited tumor burden approximately 39% in a transgenic murine model of androgen-independent prostate cancer (13). Because of its efficacy in murine tumor models, 2-ME2 is being evaluated in Phase I and Phase II clinical trials against a variety of human tumors. 2-ME2 is a promising new agent in the arsenal against cancer, yet its mechanisms of action are unclear and are likely to be multifactorial. To date, it is not known which of the effects of 2-ME2, its antiangiogenic effects or its direct effects on cancer cells, are most important for its antitumor activity. Dual involvement of antiangiogenic and cytotoxic effects may be necessary for antitumor activity by 2-ME2.

Several analogues of 2-ME2 have previously been synthesized and tested for various biological activities (11, 14–17). These studies indicate that derivatives of 2-ME2 can be designed with greater potency toward both inhibition of tumor proliferation and tubulin assembly. Modifications in the A ring led to the formation of 2-ethoxyestradiol and 2-(1-(E)-propenyl) estradiol. These two compounds were found by Cushman et al. (11) to be more potent inhibitors of tubulin polymerization and cell proliferation than 2-ME2. Analogues with modifications at the 2 position, specifically 2-(2',2',2',2',2',3'-trifluoroethoxy) and 2-ethoxy, combined with the addition of a oximino or methoxyoximino group at the 6 position on the B ring, led to compounds with increased potency for cell proliferation and tubulin polymerization (14). The conjugated estrone derivatives, 2-methoxyestrone-3-O-sulfamate and 2-ethyllestrone-3-O-sulfamate, were 10-fold more potent than 2-ME2 and caused mitotic arrest and initiation of apoptosis (15). In a small study, 2-methoxyestrone-3-O-sulfamate inhibited the growth of nitrosmothelylurea-initiated mammary tumors in rats (18). The synthesis of these analogues and their
biological evaluations with respect to the natural estrogen metabolite 2-ME2 show that specific substitutions of the A ring, either alone or in combination with specific changes in the 6 position of the B ring, lead to compounds with superior biological potency.

We recently synthesized a novel series of 2-ME2 analogues and evaluated them for activities that would predict superior antitumor properties. In contrast to the majority of the earlier work, we have a focus on the D ring of the steroid nucleus. Additionally, we have explored modifications to the A and B rings of the steroid nucleus not addressed by the previous studies. Our results show that one analogue, 14-dehydro-2-ME2, is more potent than 2-ME2. 15-Dehydro-2-ME2 is essentially equipotent, and 7-dehydro-2-ME2 and 2-ME2–15α,16α are slightly less potent than the parental compound. In one xenograft model, MDA-MB-435, three of the four analogues were tested, and all were found to have superior antitumor effects when compared with the parental compound. Basic mechanism of action studies were conducted to determine whether there are any differences among the analogues with regard to inhibition of endothelial events important in angiogenesis, inhibition of tumor cell proliferation and cytotoxicity, or microtubule disruption and initiation of signaling events common to tubulin-targeting antimicrototics. The data suggest that there are subtle differences among the analogues.

MATERIALS AND METHODS

Steroids. We chemically synthesized 2-ME2 and the novel analogues 15-dehydro-2-ME2, 14-dehydro-2-ME2, 7-dehydro-2-ME2, and 2-ME2–15α,16α. The details of the synthesis have been described recently (19).

Cell Culture. A-10 embryonic rat aortic smooth muscle cells, SK-OV-3 human ovarian carcinoma cells, HeLa human cervical carcinoma cells, and human prostate carcinoma cell lines PC3 and DU 145 were purchased from American Type Culture Collection (Manassas, VA). The human breast cancer cell line MDA-MB-435 was obtained from the Lombardi Cancer Center (Georgetown University, Washington, D.C.) and the NCI/ADR cell line was obtained from the NIH (Bethesda, MD). HUVECs were purchased from Clonetics (BioWhittaker, Walkersville, MD) and used at passages 3–6. The A-10, SK-OV-3, and HeLa cell lines were maintained in Basal Medium Eagle containing Earle’s salts (Sigma, St. Louis, MO), 50 μg/ml gentamicin, and 10% FBS (Hyclone, Logan, UT). The MDA-MB-435 and DU 145 cell lines were grown in Richter’s Medium (Biosource, Camarillo, CA) with 10% FBS and 25 μg/ml gentamicin. NCI/ADR and PC3 cells were maintained in RPMI 1640 (Biosource) containing 50 μg/ml gentamicin and 10% FBS. HUVECs were grown in EGM2 (Clonetics).

SRB Assay. The SRB assay was used to determine the sensitivity of HUVECs and various cancer cell lines to the antiproliferative and cytotoxic effects of the analogues and the parental compound, 2-ME2 (20, 21). Cells were plated at predetermined densities in 96-well plates and allowed to attach and grow for 24 h. The analogues, 2-ME2, or vehicle (ethanol) controls were added and incubated with the cells for 48 h. After drug exposure, the cells were fixed and stained with SRB, and the absorbance of the SRB solution was read at 560 nm. Dose-response curves were generated, and the concentrations of drug required to inhibit proliferation by 50% (IC50) were calculated. The IC50 was calculated for each experiment, and the mean IC50s were calculated and presented ± SE. Cytotoxicity was determined by the ability of the compound to decrease absorbance values below the levels measured at time 0, the time of drug addition (21).

Endothelial Cell Invasion. The effects of the analogues and 2-ME2 on the invasion of HUVECs were evaluated using BD Biocoat Matrigel Invasion chambers with 8-μm pores using the techniques of Wakabayashi and Nicolson (22), with minor changes. EB2M, basal medium without serum or growth factors (Clonetics), was added to the top chambers to hydrate the Matrigel-covered filters, and complete EGM2 (EB2M plus 10% FBS and growth factors) was added to the bottom chambers. HUVECs were harvested with trypsin/EDTA, washed twice with complete EGM2 medium, washed once in EB2M medium, and resuspended in invasion buffer (EBM2 containing 0.1% BSA). After a 2-h hydration, the solution in the top chamber was removed and replaced with 500 μl of invasion buffer containing 3.7 × 104 HUVECs. The chemoattractant was complete EGM2 growth medium in the bottom chamber. Drug or vehicle was added, and invasion was evaluated after 24 h. The filters were fixed with 10% formaldehyde, and cells remaining in the top chamber were removed. Cells on the lower surface of the filter were stained with H&E and counted in 10 microscope fields using a ×40 objective.

In Vivo Antitumor Activity. The antitumor effects of the analogues were compared with the antitumor effects of 2-ME2 in a xenograft model using the MDA-MB-435 human tumor cell line. Dose tolerance tests were used to determine the appropriate dose ranges for each of the analogues and 2-ME2. Female athymic NCr-nu mice (SWF-1) weighing approximately 24 g were implanted subcutaneously with 30 mg tumor fragments harvested from nude mice hosts. When the tumors reached an approximate size of 175 mg (13 days after implantation), animals were randomized to treatment or control groups, and treatments were begun. All treatments were administered on the basis of individual animal body weights. Animal weight and tumor dimensions were measured twice weekly with calipers, and tumor weights were estimated using two-dimensional measurements of length and width and the formula \[ l \times (w/2)^2 \times 0.636. \] Treatment groups contained 10 mice, and the vehicle-treated control group consisted of 20 mice. The analogues and 2-ME2 were administered i.p. daily for 30 days.

Indirect Immunofluorescence. A-10 cells were plated on glass coverslips in 24-well tissue culture plates, allowed to attach and grow, and then treated with drugs as indicated. After incubation, the cells were fixed with methanol, blocked with a 10% calf serum solution, and incubated for 90 min with a β-tubulin antibody (T-4026; Sigma). After incubation with the primary antibody and a series of washes, the cells were incubated with FITC-conjugated sheep antimouse IgG (F-3008; Sigma) for 1 h. The coverslips were washed, stained with 0.1 μg/ml DAPI for 10 min, and examined and photographed using a Nikon E800 Eclipse fluorescence microscope.

Tubulin Assembly. The effects of the analogues and 2-ME2 on the polymerization of purified bovine brain tubulin with gynecol were evaluated by the change in turbidity. Lyophilized tubulin (TL238; Cytoskeleton, Denver, CO) was solubilized in ice-cold G-PGM buffer [80 mM PIPES (pH 6.9), 1 mM MgCl2, 1 mM EGTA, and 1 mM GTP] with a final concentration of 5% gynecol and added to duplicate wells containing various concentrations of the analogues, 2-ME2, or vehicle and mixed well. The final concentration of tubulin was 3 mg/ml, and the gynecol concentration was 0.5%. The tubulin assembly was monitored at A436 nm for 60 min at 37°C. Various concentrations of the steroids were evaluated for effects on tubulin assembly. The percentage inhibition of tubulin assembly with each concentration of steroid was determined from the plateau values obtained at 45 min. Dose-response curves were generated, and the IC50 values (the concentration of drug required to inhibit tubulin assembly 50%) were calculated for each experiment and presented as the mean ± SE.

Western Blotting. MDA-MB-435 cells were treated with the approximate IC50 concentration as determined from the dose-response curves. Cells were treated for 24 h, washed, and lysed, and cellular proteins were extracted in RIPA buffer in the presence of protease inhibitors. The protein concentrations of the samples were determined with Comassie6 Plus (Pierce, Rockford, IL), and cell lysates containing equal amounts of protein were separated by SDS-PAGE, transferred to Immobilon, probed with antibodies, and detected using enhanced chemiluminescence reagents. The Bcl-2 antibody was obtained from BD PharMingen (San Diego, CA), and the phospho-Raf, phospho-ERK1/2 and phospho-p38MAPK antibodies were from Cell Signaling (Beverly, MA).

Caspase 3 Activity. Caspase 3 activity was measured by cleavage of the caspase substrate Ac-DEVD-pNA using the BIOMOL Caspase 3 Cellular Activity Kit (AK-703). MDA-MB-435 cells were incubated with the analogues, 2-ME2, or vehicle for various times. After incubation, the cells were harvested, washed, and lysed, and the cell lysates were frozen at −70°C. The protein concentration of the lysates was determined with Comassie6 Plus (Pierce), and samples containing 30 μg of protein were incubated in duplicate with the substrate DEVD-pNA at 37°C. The cleavage of the caspase 3 substrate Ac-DEVD-pNA was measured at 405 nm in a microtiter plate reader, and the specific activity was determined from the slope.

Statistical Analysis. For the inhibition of proliferation experiments, the log dose-response curves were plotted. Response was regressed on log dose using a four-parameter nonlinear model (Sigma Plot 2000 or Softmax Pro 3.1.2). The concentration of drug causing 50% inhibition of cellular proliferation was...
Novel Analogues of 2-ME2. The chemical structure of 2-ME2 is amenable to structural modification, and many analogues of 2-ME2 have been synthesized and tested for antiproliferative activities as well as the ability to inhibit tubulin assembly. Most of the modifications made previously have been in the A and B rings of the steroid nucleus (11, 14–17). We synthesized a series of analogues of 2-ME2 with structural modifications to the A, B, and D rings alone, as well as some with simultaneous modifications to more than one of the rings. To date, 18 analogues of 2-ME2 have been synthesized at this institution and tested for antiproliferative activities (19). The four analogues with most promising biological activities were further evaluated, and the results are presented herein. Interestingly, three of these four analogues resulted from modifications to the D ring alone. The chemical structures of these novel analogues and the parental structure, 2-ME2, are shown in Fig. 1.

**Endothelial Cell Effects as Predictors of Antiangiogenic Activity.** While antiangiogenic activity cannot be evaluated in vitro, there are in vitro assays that predict in vivo antiangiogenic activity. The ability of drugs to inhibit endothelial cell proliferation and endothelial cell invasion through a basement membrane material such as Matrigel are predictive of antiangiogenic activity. The antiproliferative and anti-invasive effects of 2-ME2 and the analogues were evaluated in HUVECs. The dose-response curves for the analogues and 2-ME2 are presented in Fig. 2. The corresponding IC₅₀ values for inhibition of HUVEC proliferation for these analogues ranged from 0.05 to 7.14 μM and are presented in Table 1. The results show that 14-dehydro-2-ME2 is the most potent of the steroids tested for inhibition of endothelial cell growth, with an IC₅₀ of 0.05 μM. 15-Dehydro-2-ME2 was equipotent with 2-ME2, and 7-dehydro-2-ME2 and 2-ME2-15α,16α were less potent against HUVECs when compared with 2-ME2.

The analogues were also evaluated and compared with 2-ME2 for inhibition of endothelial cell invasion in BD Biocoat Matrigel invasion chambers. The most potent analogue for inhibition of HUVEC invasion was 14-dehydro-2-ME2 (Fig. 3). 2-ME2 and 15-dehydro-2-ME2 were equipotent, and 7-dehydro-2ME2 and 2-ME2-15α, 16α were less potent in this assay. The IC₅₀ for inhibition of HUVEC invasion were calculated and are presented in Table 1. As predicted from the dose-response curves, 2-ME2 and 15-dehydro-2-ME2 were equipotent toward both endothelial cell proliferation and invasion. Some differences were seen when comparing the IC₅₀ values for the parental compound, 14-dehydro-2-ME2, and 2-ME2-15α,16α. 14-Dehydro-2-ME2 was 15 times more potent than 2-ME2 toward inhibition of proliferation and only 6-fold more potent in the anti-invasion assay. In contrast, 2-ME2-15α,16α required approximately 9-fold more compound than 2-ME2 to inhibit HUVEC proliferation and invasion. These data suggest that there may be subtle differences...
among the analogues as compared with 2-ME2 with respect to their effect on endothelial events.

**Antiproliferative Activities against Human Tumor Cell Lines.**
As an antitumor agent, 2-ME2 is thought to have a dual mechanism of action in that it inhibits angiogenesis by direct effects on endothelial cells and exerts antitumor effects by direct antiproliferative and cytototoxic effects on cancer cells. The sensitivity of several different types of cancer cells to the novel analogues was evaluated using the SRB assay. MDA-MB-435 breast cancer cells are estrogen receptor negative, vimentin positive, invasive in xenograft models (25), and contain mutant p53 (26). 2-ME2 has previously been shown to be effective against this cell line in vitro (11) and in vivo (8). The effects of the steroids were evaluated, and the dose-response curves are shown in Fig. 4. The most potent analogue was 14-dehydro-2-ME2, and 15-dehydro-2-ME2 was slightly more potent than 2-ME2. The IC_{50}s for inhibition of proliferation are presented in Table 2. All of the analogues and 2-ME2 were cytotoxic in MDA-MB-435 cells at concentrations above 7.5 μM.

The activities of the analogues were also evaluated in the Pgp-overexpressing multidrug-resistant NCI/ADR cell line. Drugs that can circumvent resistance mechanisms such as Pgp may have advantages in cancer therapy. The NCI/ADR cell line was very sensitive to all of the analogues and the parental compound (Fig. 4; Table 2), suggesting that none of the compounds is a substrate for transport by Pgp.

The potency and efficacy of the analogues were tested in two prostate cancer cell lines, PC3 and DU 145. PC3 is relatively drug sensitive, whereas DU 145 is more resistant. The dose-response curves for these cell lines show similar potency trends for the analogues that were seen in the other two cancer cell lines, yet differences in cytotoxic efficacy were observed (Fig. 4). In both prostate cancer cell lines, 15-dehydro-2-ME2 was more effective in that it reduced the absorbance values to <10% of control. This inhibition was greater than that induced by any of the other analogues or the parental compound at the concentrations tested. These effects were not observed in any of the other cell lines tested and suggest that 15-dehydro-2-ME2 should be further evaluated for superior activity against prostate models.

Cytotoxic concentrations were reached with all of the analogues in all four tumor cell lines. The analogues were also evaluated in HeLa and SK-OV-3 cells, and the IC_{50}s were calculated and compared with 2-ME2 (Table 2). Consistent with the effects in the other cell lines, the analogues and 2-ME2 caused concentration-dependent inhibition of cell growth of the estrogen-dependent MCF7 cell lines (data not shown).

In summary, the data for inhibition of tumor cell proliferation indicate that 14-dehydro-2-ME2 is more potent than 2-ME2 in five of the six cell lines tested, and 15-dehydro-2-ME2 is approximately equipotent with 2-ME2, with the exception of the DU 145 cell line, in which the novel analogue is more potent and effective than the parental compound. The 7-dehydro-2-ME2 and 2-ME2-15α,16α analogues are slightly less potent than 2-ME2 in all of the cancer cell lines tested.

**In Vivo Antitumor Activity.** Three analogues were tested and compared with 2-ME2 in a murine xenograft tumor model. The MDA-MB-435 model was used because the cell line was sensitive to our analogues in vitro and because in prior in vivo studies, conducted by Klauber et al. (8), 2-ME2 had antitumor activity in a MDA-MB-435 xenograft model. Dose tolerance tests were conducted with the analogues and 2-ME2 to determine the appropriate doses for the antitumor trial. No frank toxicity was reached for any of the compounds, and the doses chosen for the efficacy trial caused no adverse effects as indicated by weight loss in either the tolerance test or the efficacy trial. MDA-MB-435 tumor fragments (30 mg) were implanted and allowed to grow until they reached an approximate size of 175 mg, and treatment was initiated. The mice were treated daily for 30 days, and the tumor volumes were estimated twice weekly. The tumor burden was plotted versus time, and the results are shown in Fig. 5.

Statistical analyses of the in vivo trial were conducted, and the results are presented in Table 3. The tumor growth of each animal in the trial was mathematically modeled to determine total tumor load as defined as the area under the curve. These data indicate that the average area for each treatment group is significantly different from the control. Treatment with 15-dehydro-2-ME2, 14-dehydro-2-ME2, and 2-ME2-15α,16α resulted in a statistically significant, smaller tumor load than that in the control group, with respective inhibitions of 15.5%, 29.4%, and 26.7% as compared with control. Treatment with 2-ME2 led to a slightly larger total tumor load. This reflects the variable nature of the growth curve in this treatment group, which increases rapidly initially and then flattens around day 30. In summary, 15-dehydro-2-ME2-, 14-dehydro-2-ME2-, and 2-ME2-15α,16α-treated tumors provided statistically significant antitumor effects, yet 2-ME2 did not provide any antitumor effects in this trial at a dose of either 37.5 (Fig. 5; Table 3) or 75 mg/kg/day (data not shown). All three of the new analogues tested, 15-dehydro-2-ME2, 14-dehydro-2-ME2, and 2-ME2-15α, 16α, were superior to 2-ME2 in this trial.

**Disruption of Mitotic Spindles.** The antimotic activity of 2-ME2 was first described over a decade ago (9), and the interruption of

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**Table 1** Inhibitory effects of 2-ME2 and analogues toward HUVEC proliferation and invasion

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC_{50} (μM) HUVEC proliferation</th>
<th>IC_{50} (μM) HUVEC invasion</th>
</tr>
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<tbody>
<tr>
<td>2-ME2</td>
<td>0.77 ± 0.10</td>
<td>1.78 ± 0.17</td>
</tr>
<tr>
<td>2-ME2-15α,16α</td>
<td>0.05 ± 0.00</td>
<td>0.29 ± 0.04</td>
</tr>
<tr>
<td>15-dehydro-2-ME2</td>
<td>0.88 ± 0.04</td>
<td>1.98 ± 0.01</td>
</tr>
<tr>
<td>14-dehydro-2-ME2</td>
<td>0.57 ± 0.50</td>
<td>5.15 ± 0.50</td>
</tr>
<tr>
<td>7-dehydro-2-ME2</td>
<td>ND*</td>
<td>17.48 ± 1.12</td>
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</table>

*ND, not determined.*

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**Fig. 3.** Effects of 2-ME2 and analogues on cell invasion of HUVECs. BD Biocoat Matrigel invasion chambers were used to define the dose-response curves of the drugs for invasion of HUVECs. Data represent the means of three experiments ± SE.
mitosis leading to initiation of apoptosis appears to be an important part of the mechanism of action of 2-ME2. We investigated the dose-dependent effects of the analogues on mitotic and interphase microtubules in A-10 cells by indirect immunofluorescent techniques. The mitotic spindle is affected by antimitotics at concentrations that do not affect interphase microtubules because the mitotic spindle is more dynamic than interphase microtubules (27). Consistent with the effects of other antimitotics, the first effects observed, with the lowest concentrations of drugs tested, were the formation of abnormal mitotic spindles (Fig. 6). Normal bipolar spindles were observed in vehicle-treated cells (Fig. 6A), and abnormal mitotic spindles were seen in the 2-ME2- and analogue-treated cells (Fig. 6, B–F). Abnormal spindles were associated with dysfunctional alignment of the DNA. 2-ME2 initiated the formation of abnormal bipolar spindles with very short spindle fibers (Fig. 6B). The effect on the length of the spindle was observed in cells treated with each of the analogues (Fig. 6, C–F). This effect differs from other microtubule-depolymerizing anti-mitotics, such as the Vinca alkaloids, cryptophycins (28), and symplostatin 1 (29). These antimitotics all cause the formation of multipolar spindles with normal-length spindle fibers and differ from the analogues and 2-ME2 with regard to the length of the spindle fibers. Cells with abnormal numbers of spindle poles are observed after treatment with the analogues (Fig. 6, D–F), and, as can be visualized in these pictures, the mitotic spindles are consistently very short. With higher doses, the spindles are identified only by tiny spots of tubulin in a cytoplasm devoid of any other microtubules. This effect of 2-ME2 and the novel analogues is quite distinct from those of other microtubule-depolymerizing agents that we have studied.

Because the formation of abnormal mitotic spindles is associated with mitotic arrest, the effects of the analogues on cell cycle progression were evaluated by flow cytometry. MDA-MB-435 cells were treated at various times with the approximate IC_{50} concentration for inhibition of proliferation. The analogues and parental compound initiated mitotic arrest within 18 h of treatment, consistent with their disruptive effects on the mitotic spindles (data not shown).

Table 2. The IC_{50} for inhibition of proliferation of drug-sensitive and multidrug-resistant cancer cell lines

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>IC_{50} (μM)</th>
</tr>
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<tbody>
<tr>
<td>MDA-MB-435</td>
<td>1.40 ± 0.23</td>
</tr>
<tr>
<td>NCI/ADR</td>
<td>0.42 ± 0.03</td>
</tr>
<tr>
<td>PC3</td>
<td>2.23 ± 0.10</td>
</tr>
<tr>
<td>DU 145</td>
<td>3.50 ± 0.42</td>
</tr>
<tr>
<td>HeLa</td>
<td>0.42 ± 0.03</td>
</tr>
<tr>
<td>SK-OV-3</td>
<td>1.53 ± 0.14</td>
</tr>
<tr>
<td>2-ME2</td>
<td>1.48 ± 0.07</td>
</tr>
<tr>
<td>15-dehydro-2-ME2</td>
<td>0.02 ± 0.00</td>
</tr>
<tr>
<td>14-dehydro-2-ME2</td>
<td>0.08 ± 0.01</td>
</tr>
<tr>
<td>7-dehydro-2-ME2</td>
<td>6.09 ± 2.56</td>
</tr>
<tr>
<td>2-ME2-15α, 16α</td>
<td>6.09 ± 2.56</td>
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</tbody>
</table>

IC_{50} values for inhibition of proliferation were calculated by nonlinear regression analysis (n = 3, mean ± SE).

ND, not determined.
Disruption of Cellular Microtubules. The concentration-dependent effects of the analogues and 2-ME2 on interphase microtubules were examined. The effects of the analogues on interphase microtubules resemble those of all other microtubule-depolymerizing agents that we have studied. The first effects on interphase microtubules are the loss of microtubules at the periphery of the cell and the appearance of “relaxed microtubules” that are less rigid than the microtubules in vehicle-treated cells while maintaining normal cell size and shape (Fig. 7). Disruption of interphase microtubules occurred at concentrations higher than those required to cause abnormal mitotic spindles (Figs. 6 and 7). Concentration-dependent loss of the interphase microtubules reflected the potency toward inhibition of proliferation. 15-Dehydro-2-ME2 and 2-ME2 were equipotent at inducing microtubule loss (Fig. 7, B and C). Approximately 50% microtubule loss was observed between 5 and 10 μM; 75 μM concentrations were required for 95% microtubule loss with these two compounds. 14-Dehydro-2-ME2 was more potent than 2-ME2, with 50% cell loss at approximately 0.5 μM (Fig. 7D) and 95% depolymerization at 5 μM. 7-Dehydro-2-ME2 and 2-ME2-15α,16α are slightly less potent than 2-ME2, with approximately 50% interphase microtubule loss at 25 μM (Fig. 7, E and F) and 95% depolymerization at 100 μM.

Inhibition of Tubulin Assembly. The effects of the analogues on the assembly of purified bovine brain tubulin were evaluated. Previous studies have shown that 2-ME2 inhibits the polymerization of tubulin, and our results concur with these findings. In addition to 2-ME2, 15-dehydro-2-ME2, 14-dehydro-2-ME2, and 2-ME2-15α,16α inhibited the assembly of tubulin in a concentration-dependent manner. The inhibitory effects of 14-dehydro-2-ME2 are shown in Fig. 8. This figure is representative of results obtained with the other compounds. As anticipated from the other biological activities of 15-dehydro-2-ME2, this compound was essentially equipotent with 2-ME2 for inhibition of tubulin assembly with respective EC50s of 19.9 and 16.6 μM (Table 4). These two compounds were essentially equipotent with regard to both inhibition of tubulin polymerization (Table 4) and microtubule depolymerization (Fig. 7). In the tubulin polymerization assay, 14-dehydro-2-ME2 was more potent than 2-ME2, with an EC50 of 2.5 μM. Comparing the EC 50 s for inhibition of tubulin polymerization, 14-dehydro-2-ME2 is 8-fold more potent than 2-ME2 in this assay, and yet it is >20-fold more potent than

Table 3. Determination of statistically significant antitumor activity

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Average area (log mg)</th>
<th>SE</th>
<th>P</th>
<th>Average area (mg) (% inhibition of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.8126</td>
<td>0.0597</td>
<td>&lt;0.0001</td>
<td>333.5 (0)</td>
</tr>
<tr>
<td>2-ME2</td>
<td>5.8265</td>
<td>0.0842</td>
<td>&lt;0.0001</td>
<td>333.5 (0)</td>
</tr>
<tr>
<td>15-dehydro-2-ME2</td>
<td>5.6465</td>
<td>0.0842</td>
<td>&lt;0.0001</td>
<td>282.3 (15.4)</td>
</tr>
<tr>
<td>14-dehydro-2-ME2</td>
<td>5.4670</td>
<td>0.0859</td>
<td>&lt;0.0001</td>
<td>235.7 (29.4)</td>
</tr>
<tr>
<td>2-ME2-15α,16α</td>
<td>5.5036</td>
<td>0.0842</td>
<td>&lt;0.0001</td>
<td>244.6 (26.7)</td>
</tr>
</tbody>
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* P comparing average area for treatment group with that for control group, based on an F-test.
2-ME2 in its ability to depolymerize microtubules in cells (Fig. 7, B and C). 2-ME2-15α,16α was less potent for inhibition of tubulin assembly than 2-ME2 and the other analogues tested, consistent with its antiproliferative and microtubule-depolymerizing effects in cells. It is interesting to note that for inhibition of tubulin polymerization, over 7 times more 2-ME2-15α,16α was required to inhibit tubulin assembly 50% when compared with 2-ME2. However, only 2.5-fold more 2-ME2-15α,16α was required to cause 50% loss of cellular microtubules. It appears that there is not a simple relationship between the inhibition of tubulin assembly in vitro and the in vivo loss of cellular microtubules for all of these analogues when compared with 2-ME2.

The ability of the analogues to inhibit tubulin assembly in the absence of microtubule-associated proteins suggests that they interact directly with tubulin. In the tubulin assembly assay, the final concentration of tubulin was 3 mg/ml (30 μM). 2-ME2, 15-dehydro-2-ME2, and 14-dehydro-2-ME2 were effective at substoichiometric concentrations, and 2-ME2-15α,16α required superstoichiometric concentrations to inhibit tubulin assembly.

Nuclear Changes and Caspase 3 Activation. A hallmark of agents that target microtubules, including microtubule depolymerizers and microtubule stabilizers, is the formation of micronuclei. 2-ME2 and all of the analogues caused these characteristic effects in HeLa (data not shown) and A-10 cells (Fig. 9). In contrast to the normal rounded intact nucleus (Fig. 9A), 2-ME2 induces extensive micronucleation (Fig. 9B), and similar effects are initiated by the novel analogues (Fig. 9, C–F). It is interesting to note that not all cells are affected. Micronucleation is independent of mitotic arrest in both HeLa and A-10 cells. The micronucleation effects suggest that the analogues initiate an apoptosis cascade.

A late event in apoptosis is the activation of caspase 3, a caspase involved in the destruction of the cell. The time course of caspase activation was evaluated in MDA-MB-435 cells treated with the approximate IC₈₀ concentration for inhibition of proliferation for selected time periods. All of the analogues and 2-ME2 activated the caspase cascade in a time-dependent manner consistent with induction of apoptosis (Fig. 10). Regression lines of caspase activity plotted against exposure time were fit for each steroid. All slopes were positive and statistically different from control, indicating that all of the analogues and 2-ME2 caused an increase in caspase activity over time. The slopes varied, however, with 15-dehydro-2-ME2 and 2-ME2-15α,16α displaying the largest increases. Western blots detecting activated caspase 3 confirmed caspase activation of 2-ME2 and the analogues at 24 h (data not shown).

Activation of Signal Transduction Pathways. One characteristic of apoptosis induced by microtubule-targeting agents is the phosphorylation of the antiapoptotic protein Bcl-2 (30). The ability of the novel analogues to initiate Bcl-2 phosphorylation was investigated. MDA-MB-435 cells were treated for 24 h with the IC₈₀ concentration of 2-ME2, the analogues, or vehicle. Cell lysates containing 30 μg of

2-ME2 and analogues on mitotic spindles. Mitotic spindles were visualized in A-10 cells by indirect immunofluorescence techniques after an 18-h treatment with vehicle (A), 1 μM 2-ME2 (B), 1 μM 15-dehydro-2-ME2 (C), 0.1 μM 14-dehydro-2-ME2 (D), 5 μM 7-dehydro-2-ME2 (E), and 5 μM 2-ME2-15α,16α (F).
qprotein were separated by PAGE and then transferred to Immobilon membranes and probed for Bcl-2 and for activated proteins in the MAPK pathways. The appearance of the slower migrating form of Bcl-2 consistent with its phosphorylation was seen after treatment with 2-ME2 and all of the analogues tested, although some differences were noted among the analogues (Fig. 11). 14-Dehydro-2-ME2 (Fig. 11, Lane 3) initiated the formation of two slower migrating bands, although the third band is very light. This is consistent with the phosphorylation of multiple sites on Bcl-2. The levels of phosphorylated and unphosphorylated Bcl-2 appear to be equal for 15-dehydro-2-ME2 and 2-ME2-15α,16α (Fig. 11, Lanes 4 and 5) and differ from those of 2-ME2 (Lane 2), where the level of unphosphorylated Bcl-2 is greater than the levels of phosphorylated Bcl-2. While all of the analogues and 2-ME2 initiated the phosphorylation of Bcl-2, consistent with the effects of other antimitotics, there appear to be subtle differences among the analogues with respect to Bcl-2 phosphorylation.

The effects of the compounds on the phosphorylation and activation of Raf, ERK1/2, and p38MAPK were evaluated using activation (phosphorylation)-specific antibodies. The results concur with the results observed with Bcl-2 and suggest that there are some differences among the analogues. After 24 h, the activation of Raf was similar among all of the compounds and did not appear to be different than the controls (Fig. 11). However, the activation of ERK1/2 was more pronounced in cells treated with 14-dehydro-2-ME2 (Lane 3) when compared with 2-ME2 and the other analogues. In contrast, the activation of p38MAPK was less in the 14-dehydro-2-ME2-treated cells (Lane 3) when compared with the other 2-ME2 compounds. These subtle differences in activation states of signaling proteins were
confirmed with another set of lysates (data not shown). These data suggest that the analogues activate the same signaling pathways as 2-ME2 but that there are slight differences among the analogues in this activation.

**Selectivity of the Analogues.** Angiogenesis is an extremely complex process requiring coordination of endothelial cell proliferation, invasion, and migration. To estimate whether there are differences among the analogues with respect to specificity toward endothelial events, a series of ratios were calculated from the data presented in Tables 1 and 2. A ratio for 2-ME2 and each analogue was calculated by dividing the IC50 for HUVEC invasion by the IC50 for HUVEC proliferation. If the calculated ratio is 1, then that would suggest that both endothelial cell processes, proliferation and invasion, are equally sensitive. A calculated ratio greater than 1 would indicate that more drug is required to inhibit invasion than is required to inhibit proliferation. Ratios were calculated and are shown in Table 5. With respect to HUVEC invasion and proliferation, 2-ME2, 15-dehydro-2-ME2, and 2-ME2-15α,16α are identical with ratios of 2.31, 2.25, and 2.45, respectively, suggesting similar sensitivity of these three agents toward these endothelial processes, i.e., it requires approximately 2.5-fold more drug to inhibit HUVEC invasion than is required to inhibit HUVEC proliferation. In contrast, the ratio for 14-dehydro-2-ME2 is 5.80; thus, it requires approximately 6-fold more 14-dehydro-2-ME2 to inhibit HUVEC invasion than is required to inhibit proliferation. These data indicate that when compared with the parental compound and the other two analogues (15-dehydro-2-ME2 and 2-ME2-15α,16α), relatively more 14-dehydro-2-ME2 is required to inhibit HUVEC invasion than proliferation. These ratios suggest that although the analogues cause inhibition of both endothelial cell proliferation and invasion, there are some differences among the analogues and 2-ME2 with respect to endothelial effects important in angiogenesis.

2-ME2 differs from most of the antiangiogenic agents under clinical development in that it has antiangiogenic activity and direct antiproliferative and cytotoxic effects against tumor cells. To date, it is not known whether either activity is more critical for the antitumor effects of 2-ME2. To estimate differences in the selectivity of the analogues toward inhibition of HUVECs versus tumor cell proliferation, ratios were calculated from the data presented in Tables 1 and 2 for the analogues and 2-ME2 by dividing the IC50 for HUVEC proliferation by the IC50 for inhibition of SK-OV-3 proliferation. If a
Analogue of 2-ME2 were designed, synthesized, and tested for biological properties that predict anticancer effects. The major focus of the structural modifications was the D ring of the parental compound, although one analogue was designed with a modification in the B ring. The results of this study show that modifications in the D ring of 2-ME2, specifically the unsaturation of the D ring in the 14 position (14-dehydro-2-ME2), can lead to increased potency for biological events important in the control of cancer. However, the specific location of the unsaturation within the D ring is critical because, in contrast to the unsaturation in the 14 position, unsaturation in the 15 position does not increase the potency as compared with the parental compound. The addition of the acetonide moiety between the 15 and 16 positions on the D ring reduced the potency of the compound slightly when compared with 2-ME2 but provided subtle changes in the selectivity of the compound for different biological effects. While others have clearly shown that the structures of various groups on the A ring are critical for biological activities, our results show that the D ring of the steroid nucleus also plays a key role in potency and biological activity.

Although much importance has been placed on the increased potency of these novel 2-ME2 analogues, the apparent differences among analogues with regard to specificity and mechanism of action should not be neglected. The clinical work with the Vinca alkaloids suggests that increased potency may not be the most important consideration for in vivo efficacy and safety. Our work with these new analogues of 2-ME2 shows that potency is not the most important consideration. The two analogues with the best antitumor activity were the most and least potent of the series. For these reasons, we investigated other differences besides potency to better understand what properties are important for in vivo efficacy. We are synthesizing and testing additional new 2-ME2 analogues. Identification of the biological characteristics of the analogues that can predict superior antitumor activities will streamline the selection of the most promising 2-ME2 derivatives for clinical development.

2-ME2 is an antiangiogenic agent with direct activity on developing vasculature. While 2-ME2 is an effective inhibitor of rapidly dividing endothelial cells, it has no effect on confluent cultures (6). In several models of angiogenesis, including tumor angiogenesis, 2-ME2 was an effective inhibitor (6, 8, 31). Our novel analogues are effective inhibitors of endothelial cell activities that predict antiangiogenic activity. The value of these in vitro assays is reflected in the fact that they were recently initiated at the National Cancer Institute Developmental Therapeutics Branch to identify agents with potential antiangiogenic activity. Our studies evaluated the effects of the analogues on HUVECs. It is well known that endothelial cells can differ depending on their vascular bed of origin. Early studies evaluated the antiproliferative effects of 2-ME2 and other estrogen metabolites against microvascular endothelial cells of various origins and large vessel endothelial cells including HUVECs (6). The authors concluded that

**Table 5** Ratios of the mean IC$_{50}$s for inhibition of proliferation and cell invasion were calculated from the dose-response curves

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC$_{50}$ HUVEC invasion</th>
<th>IC$_{50}$ HUVEC proliferation</th>
<th>IC$_{50}$ SK-OV-3 proliferation</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-ME2</td>
<td>2.31</td>
<td>0.50</td>
<td>1.22</td>
</tr>
<tr>
<td>15-dehydro-2-ME2</td>
<td>2.25</td>
<td>0.82</td>
<td>1.58</td>
</tr>
<tr>
<td>14-dehydro-2-ME2</td>
<td>5.80</td>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td>7-dehydro-2-ME2</td>
<td>ND*</td>
<td>1.22</td>
<td></td>
</tr>
<tr>
<td>2-ME2-15a,16x</td>
<td>2.45</td>
<td></td>
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* ND, not determined.

**Fig. 10.** The effect of 2-ME2 and the analogues on caspase 3 activation. MDA-MB-435 cells were treated with the IC$_{50}$ concentration, 10 μM 2-ME2, 10 μM 15-dehydro-2-ME2, 1 μM 14-dehydro-2-ME2, and 10 μM 2-ME2-15a,16x for 0–48 h, and cell lysates were prepared. The lysates were evaluated for the ability to cleave the caspase 3 substrate Ac-DEVD-pNA. All of the steroids tested provided statistically significant increases in caspase activity over time (P < 0.05). n = 3–4 ± SE.

**Fig. 11.** The effects of the steroids on Bcl-2 phosphorylation and the activation of Raf, ERK1/2, and p38 MAPK. MDA-MB-435 cells were treated with vehicle (Lanes 1 and 6) or the IC$_{50}$ concentrations for inhibition of proliferation [10 μM 2-ME2 (Lane 2), 1 μM 14-dehydro-2-ME2 (Lane 3), 10 μM 15-dehydro-2-ME2 (Lane 4), and 10 μM 2-ME2-15a,16x (Lane 5)] for 24 h, and cell lysates were prepared in RIPA buffer. Aliquots containing equal amounts of protein were separated by PAGE, transferred to Immobilon, and probed with the specific antibodies indicated.

**DISCUSSION**

A compound is more potent against endothelial cells than the cancer cells, the ratio will be less than 1. The results indicate that 2-ME2, 15-dehydro-2-ME2, and 14-dehydro-2-ME2 have similar ratios, ranging from 0.50 to 0.82, and these ratios indicate a potential selectivity toward HUVECs when compared with a cancer cell line. The ratio of 7-dehydro-2-ME2 was equal to 1.22 in the SK-OV-3 cells, potentially indicating less endothelial cell specificity as compared with cancer cell lines. In five of the six tumor cell lines examined, 2-ME2-15a,16x had a substantially higher ratio than the other compounds, indicating that it may have selectivity toward tumor cell lines over endothelial cell lines. This is interesting because 2-ME2-15a,16x had better antitumor activity than 2-ME2 and 15-dehydro-2-ME2. The ratios indicate that there are interesting differences among the analogues and 2-ME2 with regard to in vitro effects and that these relationships should be further examined to predict which analogues have superior antitumor effects.
the inhibitory effects of 2-ME2 were essentially the same with several different types of endothelial cells (6). Thus, the effective inhibition of our novel analogues of the proliferation and invasion of HUVECs may be indicative of inhibitory activity in microvascular endothelial cells of a developing tumor as well.

The direct cytotoxic effects of 2-ME2 are also thought to be important to its mechanism of action. 2-ME2 has broad antiproliferative activities against many types of human tumor cell lines (11). Our analogues are also effective against a range of tumor cell types tested including breast, prostate, ovarian, and cervical cancer. The NCI 60 cell line data suggest that breast cancer cells appear to be particularly sensitive to 2-ME2, and clinical trials evaluating 2-ME2 in breast cancer are ongoing. Likewise, the analogues are effective against both estrogen receptor-negative, p53-mutant breast cancer cells (MDA-MB-435) and estrogen receptor-positive breast cancer cells with wild-type p53 (MCF7) cells in vitro and MDA-MB-435 cells in vivo. In addition to breast cancer, the clinical activity of 2-ME2 is being evaluated against prostate cancer. Recent reports show that 2-ME2 is effective against both androgen-dependent and androgen-independent human prostate cell lines in vitro (13, 32) and against a spontaneous androgen-independent murine prostate tumor in vivo (13). Our novel analogues had antiproliferative activity against both DU 145 and PC3 prostate cancer cell lines. The excellent antiproliferative and cytotoxic effects of 15-dehydro-2-ME2 against these two cell lines were particularly striking and suggest that this analogue may be superior to 2-ME2 in prostate models.

In this trial, no antitumor efficacy was observed with 2-ME2. These data are in contrast to the results obtained by Klauber et al. (8) using the same tumor. The differences in efficacy of 2-ME2 in the trials may be due to several factors. The tumors in our trial were passaged in a murine host rather than in vitro cell culture. The previous study used an initial tumor burden of \(1 \times 10^6\) cells, and our trial was initiated with a much larger 30-mg tumor fragment. Consequently, we evaluated a later stage tumor, as indicated by the tumor burden at 40 days in control animals, which was >2.5 times the tumor burden reported in the previous study. Also the growth rate of the tumors in the two trials differed substantially, with an approximate doubling time of 6.5 days in our trial and 9 days in the previous trial. Differences may relate to the phenotype of the specific tumor sample. Considerable variations may occur between samples of the same tumor; for example, different sublines of the human prostate tumor LNCaP provided variations may occur between samples of the same tumor. The differences in efficacy of 2-ME2 in the trials may be due to several factors. The tumors in our trial were passaged in a murine host rather than in vitro cell culture. The previous study used an initial tumor burden of \(1 \times 10^6\) cells, and our trial was initiated with a much larger 30-mg tumor fragment. Consequently, we evaluated a later stage tumor, as indicated by the tumor burden at 40 days in control animals, which was >2.5 times the tumor burden reported in the previous study. Also the growth rate of the tumors in the two trials differed substantially, with an approximate doubling time of 6.5 days in our trial and 9 days in the previous trial. Differences may relate to the phenotype of the specific tumor sample. Considerable variations may occur between samples of the same tumor; for example, different sublines of the human prostate tumor LNCaP provided variations may occur between samples of the same tumor.

A modest antitumor effect of 2-ME2 is not without precedent. In the nitrosomethylurea-induced mammary tumor model, 2-ME2 provided only 12.5% inhibition of tumor growth (18).

A considerable body of evidence suggests that the mechanisms of action of 2-ME2 are multifactorial (34). One possible mechanism of action of 2-ME2 is as an antimicrotubule agent. Although microtubule interruption is not necessarily the only mechanism of action of 2-ME2, it is clearly a defined mechanism. 2-ME2 directly inhibits tubulin assembly and inhibits colchicine binding (11, 14, 35). At concentrations that inhibit cellular proliferation, 2-ME2 inhibits mitotic progression by disrupting spindle structures (9). Interphase microtubules are less dynamic (27) and therefore less sensitive to antimicrotubotics, so higher concentrations of these drugs, including 2-ME2, are required to observe interphase microtubule disruption (36). The ability of 2-ME2 to initiate mitotic arrest and subsequent apoptosis is well documented (7, 13, 15, 37–40).

Similar studies were performed to define whether the new analogues act through disruption of microtubules as well. All of the analogues caused disruption of mitotic spindles at concentrations in the low micromolar range; in addition to disruption of cellular microtubules at concentrations slightly higher than that necessary to disrupt mitotic spindles. Each of the analogues caused mitotic arrest within 18 h of treatment, initiation of apoptosis (as visualized by the formation of micronuclei), and activation of the caspase cascade including caspase 3. Furthermore, activation of signal transduction pathways occurred and was detected by the phosphorylation of Bcl-2, Raf, ERK1/2, and p38MAPK, but interestingly, the levels of activation varied among the analogues. Thus, even though the analogues may have the same intracellular binding site (presumably tubulin), they also appear to be able to have slightly different specificities for biological processes and degrees of activation of signaling pathways. These subtle differences will be investigated further, with these and additional 2-ME2 analogues, to allow us to predict whether any of these subtle differences are important for antitumor efficacy. In conclusion, our data suggest that the mechanism of action of the 2-ME2 analogues involves an interaction with tubulin leading to interruption of the functioning of mitotic spindles, causing mitotic arrest and activation of signal transduction cascades, leading to the initiation of apoptosis.

We designed, synthesized, and tested a unique series of analogues of 2-ME2 to identify derivatives with superior antitumor effects. Three analogues tested in head-to-head comparison with 2-ME2 provided better antitumor efficacy. These tests confirm that analogues of 2-ME2 with better antitumor activity can be identified.

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Novel 2-Methoxyestradiol Analogues with Antitumor Activity
Tina L. Tinley, Rachel M. Leal, Deborah A. Randall-Hlubek, et al.


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