Significant antitumor activity in vivo following treatment with the microtubule agent ENMD-1198

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Abstract

Clinical studies using the microtubule-targeting agent 2-methoxyestradiol (2ME2; Panzem) in cancer patients show that treatment is associated with clinical benefit, including prolonged stable disease, complete and partial responses, and an excellent safety profile. Studies have shown that 2ME2 is metabolized by conjugation at positions 3 and 17 and oxidation at position 17. To define structure-activity relationships for these positions of 2ME2 and to generate metabolically stable analogues with improved anti-tubulin properties, a series of analogues was generated and three lead analogues were selected, ENMD-1198, ENMD-1200, and ENMD-1237. These molecules showed improved metabolic stability with >65% remaining after 2-h incubation with hepatic microsomes. Pharmacokinetic studies showed that oral administration of the compounds resulted in increased plasma levels compared with 2ME2. All three analogues bind the colchicine binding site of tubulin, induce G2-M cell cycle arrest and apoptosis, and reduce hypoxia-inducible factor-1α (HIF-1α) protein translation through disruption of microtubules by a mechanism that is currently under investigation (5). HIF-1α is the regulated subunit of the transcription factor HIF-1 that is expressed when cells are subjected to stress, particularly hypoxia. Stress signals cause HIF-1α protein stabilization and its nuclear translocation and association with HIF-1β. The heterodimeric transcription factor HIF-1 regulates >70 genes involved in angiogenesis, glycolysis, metastasis, and cell growth (8–10). The down-regulation of HIF-1α by 2ME2 presumably contributes to its antiangiogenic activity in vivo (5).

Clinical studies in humans and in vivo studies in rodents have shown that orally administered 2ME2 is metabolized mainly by processes similar to those reported for estradiol and other steroid hormones, with conjugation at positions 3 and 17 as well as oxidation at position 17 to form 2-methoxyestrone (Fig. 1A; refs. 11–15). The conjugated forms of 2ME2 are inactive,3 and oxidation to 2-methoxyestrone results in 10- to 100-fold loss in activity in vitro (6). 2ME2 has been reformulated, which has resulted in improved plasma drug levels in mice and humans without increased toxicity, and improved antitumor activity in preclinical models (16–18).

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2 Unpublished data.

that of cyclophosphamide. ENMD-1198 was selected as the lead molecule in this analogue series and is currently in a phase I clinical trial in patients with refractory solid tumors. [Mol Cancer Ther 2008;7(6):1472–82]
Unlike many MTA, 2ME2 is not a substrate for multidrug resistance pumps (Table 1A; refs. 1, 3). It is well tolerated in preclinical and clinical studies (16–20). In marked contrast to most other antimicrotubule drugs, 2ME2 treatment has not caused neurotoxicity or myelosuppression even in patients who have been treated for >5 years (11, 12, 14, 15). 2ME2 is therefore an attractive lead structure for discovery and development of novel MTA that may have more favorable toxicity profiles.

Clinical pharmacokinetic studies with 2ME2 suggest that a strategy directed at altering metabolism by modifying the two hydroxyl groups might be an attractive approach to improving potency and decreasing metabolism of new analogues. We generated analogues of 2ME2 with substituents at the metabolically active positions 3 and 17. These new compounds retained both antiangiogenic and antitumor activities of 2ME2. Several analogues with one or more substituent changes showed antiproliferative activity similar to or greater than that of 2ME2 (21, 22). From these series, three lead compounds were selected for further study: ENMD-1198 [3-carboxyamide-2-methoxyestra-1,3,5(10)16-tetraene], ENMD-1200 [3-carboxyamide-17-methylene-2-methoxyestra-1,3,5(10)-triene], and ENMD-1237 [3-carboxyamide 2-methoxyestra -1,3,5(10)-triene; Fig. 1B]. The present study describes the anti-tubulin properties, the antitumor activities in vitro and in vivo, and the pharmacokinetic profiles of these molecules.

**Materials and Methods**

**Reagents**

2ME2 was purchased from Tetrionics (now SAFC) and Organon. Estradiol and estrone (starting materials for the syntheses) were obtained from Aldrich. Initial synthesis of compounds was carried out at EntreMed. Large-scale synthesis of analogues was conducted by Cardinal Health (compounds were ≥95% pure with no single impurity more than 3%). The following reagents were purchased: rat anti-α-tubulin monoclonal antibody (Chemicon), anti-rat Alexa 488 (Molecular Probes), mouse anti-HIF-1α antibody (BD Transduction Laboratories), mouse anti-α-actin antibody (Sigma), anti-mouse horseradish peroxidase–conjugated antibody (Molecular Probes), and [3H]colchicine (Dupont).

**Cell Culture**

Human MDA-MB-231 breast carcinoma, MDR1/231 multidrug-resistant cells derived from MDA-MB-231 cells, U87-MG glioblastoma, and PC3 prostate cancer cells were maintained in DMEM/F-12 (Life Technologies) supplemented with 10% (v/v) fetal bovine serum (Hyclone Laboratories). Mouse Lewis lung carcinoma (LLC) cells were maintained in DMEM (Life Technologies) supplemented with 5% fetal bovine serum, glutamine, nonessential amino acids, vitamins, and sodium pyruvate. HT-29 human colon cancer cells were grown in DMEM (Bio-Whittaker) supplemented with 5% fetal bovine serum and l-glutamine. Human umbilical vein endothelial cells were grown in EGM medium (Clonetics). Cells were grown in a humidified chamber at 37°C with 5% CO₂.

**Proliferation Assays**

IC₅₀ values for 2ME2 and analogues were obtained by assessment of DNA synthesis using a bromodeoxyuridine incorporation ELISA kit assay (Roche) or by cleavage of the tetrazolium salt (WST-1) to soluble formazan dye by cellular mitochondrial dehydrogenases using the WST-1 Cell Proliferation Reagent (Roche). For bromodeoxyuridine and WST-1 proliferation assays, cells were seeded on 96-well plates at 1,000 to 5,000 per well depending on growth rate, allowed to attach overnight, and were then exposed to compounds for 48 h. For bromodeoxyuridine incorporation,
cells were incubated with the bromodeoxyuridine solution for 2 h followed by fixation, labeling, and absorbance measurement (370/492 nm) according to the manufacturer's instructions. WST-1 reagent was added to cell medium (1:10) followed by incubation for 0.5 to 4 h (depending on rate of cell metabolism) before reading absorbance at 450/650 nm. IC50 values were calculated using the mean from at least two independent experiments, each conducted in triplicate. GI50 values were obtained from their web site as an independent comparison of activity.4

Cell Cycle Analysis

MDA-MB-231 cells were seeded at 25,000 per well in 24-well plates and allowed to attach overnight, then exposed to the test compounds in triplicate at two times each compound’s IC50 value. Medium was gently removed after either 4, 8, 16, or 24 h of incubation. The cells were washed once with PBS and detached with trypsin/EDTA. The spent medium, wash buffer, and cell suspension from all three replicates were collected in a single tube and centrifuged at 500 g for 5 min. The cells were fixed with 70% ethanol overnight. Before cell cycle analysis, the fixed cells were centrifuged at 500 g for 5 min. The cell pellets were resuspended in 400 μL fluorescence-activated cell sorting buffer with 20 μg/mL propidium iodide. After 5 min at room temperature, samples were analyzed with a Guava cell analyzer. Drug concentrations: 1.4 μmol/L (2ME2), 0.4 μmol/L (ENMD-1198), 0.4 μmol/L (ENMD-1200), and 4.7 μmol/L (ENMD-1237).

NOTE: IC50 ± SD (μmol/L) based on three replicates with at least duplicate experiments obtained following 48-h incubation with increasing concentrations of compound as determined by bromodeoxyuridine uptake or (*) by cleavage of tetrazolium salt (WST-1). HUVEC, MDA-MB-231 human breast carcinoma, MDR/231 human breast carcinoma cell line derived from MDA-MB-231 and overexpressing P-glycoprotein, mouse LLC, U87-MG human glioma, PC3 human prostate carcinoma, and HT-29 human colon carcinoma. Median GI50 (μmol/L) after 48-h exposure to increasing concentrations of drug as determined by the Developmental Therapeutics Program, National Cancer Institute/NIH. Cell lines used included central nervous system, melanoma, ovarian, renal, prostate, and breast cancer lines. MDA-MB-231 cells were treated at two times the IC50 for 4, 8, 16, or 24 h, fixed with 70% ethanol in PBS, and stained with propidium iodide. Cell cycle analysis was done using a Guava cell analyzer. Drug concentrations: 1.4 μmol/L (2ME2), 0.4 μmol/L (ENMD-1198), 0.4 μmol/L (ENMD-1200), and 4.7 μmol/L (ENMD-1237).

Hepatocyte Metabolism

An assessment of the compound’s (1 μmol/L) metabolism was made using rat and human hepatocyte suspensions (106 cells/mL serum-free HCL15 culture medium; CXR Biosciences). Stock solutions were prepared in DMSO and were further diluted in culture medium. 7-Ethoxycoumarin was included as a positive control. The reaction mixtures were incubated at 37°C, and the reactions were

### Table 1.

(A) *In vitro* antiproliferative activities

<table>
<thead>
<tr>
<th></th>
<th>2ME2</th>
<th>ENMD-1198</th>
<th>ENMD-1200</th>
<th>ENMD-1237</th>
</tr>
</thead>
<tbody>
<tr>
<td>HUVEC</td>
<td>0.68 ± 0.15</td>
<td>0.13 ± 0.05</td>
<td>0.12 ± 0.06</td>
<td>1.19 ± 0.33</td>
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<tr>
<td>MDA-MB-231*</td>
<td>0.79 ± 0.08</td>
<td>0.20 ± 0.10</td>
<td>0.25 ± 0.04</td>
<td>1.86 ± 0.86</td>
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<tr>
<td>MDR/231*</td>
<td>0.79 ± 0.06</td>
<td>0.17 ± 0.07</td>
<td>0.24 ± 0.02</td>
<td>1.82 ± 0.81</td>
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<tr>
<td>LLC</td>
<td>0.88 ± 0.37</td>
<td>0.25 ± 0.00</td>
<td>0.23 ± 0.01</td>
<td>0.78 ± 0.11</td>
</tr>
<tr>
<td>U87-MG</td>
<td>1.48 ± 0.62</td>
<td>0.23 ± 0.08</td>
<td>0.25 ± 0.02</td>
<td>5.64 ± 3.94</td>
</tr>
<tr>
<td>PC3</td>
<td>1.08 ± 0.50</td>
<td>0.21 ± 0.10</td>
<td>0.25 ± 0.01</td>
<td>4.61 ± 1.76</td>
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<tr>
<td>HT-29</td>
<td>82.5 ± 16.6</td>
<td>0.44 ± 0.09</td>
<td>0.78 ± 0.11</td>
<td>3.64 ± 1.10</td>
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(B) National Cancer Institute cell screen data

<table>
<thead>
<tr>
<th></th>
<th>2ME2</th>
<th>ENMD-1198</th>
<th>ENMD-1200</th>
<th>ENMD-1237</th>
</tr>
</thead>
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<tr>
<td>Median GI50 (μmol/L)</td>
<td>2.46</td>
<td>0.10</td>
<td>0.34</td>
<td>2.22</td>
</tr>
<tr>
<td>No. cell lines tested</td>
<td>63</td>
<td>51</td>
<td>47</td>
<td>48</td>
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(C) Treatment of MDA-MB-231 cells results in G2-M cell cycle arrest and induction of apoptosis

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>2ME2</th>
<th>ENMD-1198</th>
<th>ENMD-1200</th>
<th>ENMD-1237</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Sub-G0 3.0</td>
<td>G1 41.2</td>
<td>S 16.6</td>
<td>G2-M 23.8</td>
</tr>
<tr>
<td>4</td>
<td>3.3</td>
<td>21.9</td>
<td>19.1</td>
<td>48.5</td>
</tr>
<tr>
<td>8</td>
<td>6.1</td>
<td>14.8</td>
<td>18.5</td>
<td>52.5</td>
</tr>
<tr>
<td>16</td>
<td>11.4</td>
<td>6.6</td>
<td>9.7</td>
<td>56.3</td>
</tr>
<tr>
<td>24</td>
<td>35.8</td>
<td>13.7</td>
<td>8.7</td>
<td>31.4</td>
</tr>
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</table>

NOTE: IC50 ± SD (μmol/L) based on three replicates with at least duplicate experiments obtained following 48-h incubation with increasing concentrations of compound as determined by bromodeoxyuridine uptake or (*) by cleavage of tetrazolium salt (WST-1). HUVEC, MDA-MB-231 human breast carcinoma, MDR/231 human breast carcinoma cell line derived from MDA-MB-231 and overexpressing P-glycoprotein, mouse LLC, U87-MG human glioma, PC3 human prostate carcinoma, and HT-29 human colon carcinoma. Median GI50 (μmol/L) after 48-h exposure to increasing concentrations of drug as determined by the Developmental Therapeutics Program, National Cancer Institute/NIH. Cell lines used included central nervous system, melanoma, ovarian, renal, prostate, and breast cancer lines. MDA-MB-231 cells were treated at two times the IC50 for 4, 8, 16, or 24 h, fixed with 70% ethanol in PBS, and stained with propidium iodide. Cell cycle analysis was done using a Guava cell analyzer. Drug concentrations: 1.4 μmol/L (2ME2), 0.4 μmol/L (ENMD-1198), 0.4 μmol/L (ENMD-1200), and 4.7 μmol/L (ENMD-1237).

4 http://dtp.nci.nih.gov/
terminated after 0 and 120 min by the addition of acetonitrile or, for the positive control, by \( \text{ZnSO}_4 \) and \( \text{Ba(OH)}_2 \) at 60 min. Reaction mixtures were centrifuged to remove cell debris, and the supernatants were analyzed by reverse-phase high-performance liquid chromatography with tandem mass spectrometric detection. The rates of metabolism were estimated by fitting a curve to the data for each concentration against time using GraphPad Prism Version 3.02 software (GraphPad Software). Percentage of parent compound remaining after 2 h is shown.

**Microtubule Assembly and Colchicine Binding**

Tubulin polymerization assays were performed as described previously (23). Purified bovine brain tubulin was isolated as described previously (24). In brief, tubulin purified from bovine brain was preincubated at 26°C with varying concentrations of compounds. Reaction mixtures were chilled on ice, GTP was added, and polymerization was followed at 26°C by turbidimetry at 350 nm in Beckman recording spectrophotometers. The extent of polymerization after 20 min was determined. IC\(_{50}\) values were determined graphically. Two independent assays were used to assess each compound. Percent inhibition of radio-labeled colchicine binding was done as described previously (4). Reaction mixtures contained 1 \( \mu \text{mol/L} \) tubulin, 5 \( \mu \text{mol/L} \) \([\text{3}^\text{H}]\)colchicine, and 5 or 50 \( \mu \text{mol/L} \) 2ME2 or analogue and were incubated for 10 min at 37°C.

**Microtubule Indirect Fluorescence**

MDA-MB-231 cells were plated at a density of 15,000 per well on eight-well glass slides (BD Biosciences) and were allowed to attach overnight. 2ME2 or analogues were added at 0.5 or 5 \( \mu \text{mol/L} \) in fresh medium and incubated for 18 h. Cells were fixed for 15 min at room temperature in PHEMO buffer [0.068 mol/L PIPES, 0.025 mol/L HEPES, 0.15 mol/L EGTA, 0.003 mol/L MgCl\(_2\)•H\(_2\)O, 10% DMSO with 3.7% (v/v) formaldehyde, 0.05% glutaraldehyde, and 0.5% TX-100; ref. 5]. Cells were washed with PBS and blocked for 20 min with 10% goat serum in PBS. The cells were incubated for 1 h with rat anti-tubulin monoclonal antibody (1:250, Chemicon), rinsed with PBS, and incubated for 30 min with anti-rat antibody conjugated to Alexa 488 (Molecular Probes). After rinsing, the chambers were removed and nuclei were stained with propidium iodide or 4′,6-diamidino-2-phenylindole in mounting medium (Vector Laboratories). Coverslips were applied and cells were photographed at \( \times 300 \) magnification using an Olympus 1×70 microscope equipped with a FITC filter.

**HIF-1\(a\) Western Blot Analysis**

MDA-MB-231 cells were plated on 10 cm dishes at 700,000 per dish, allowed to attach overnight, and treated for 15 h with agents in fresh medium. Hypoxic cells were incubated in a hypoxia chamber (Billups-Rothenberg) with 1% \( \text{O}_2 \) for 4 h at 37°C, whereas normoxic cells remained in the \( \text{CO}_2 \) incubator. Cells were lysed in whole-cell extract buffer [50 mmol/L Tris, 2 mmol/L EDTA, 100 mmol/L NaCl, 1% NP-40 (pH 7.5) with protease inhibitors (Roche) complete mini tab; 5 \( \mu \text{g/mL} \) aprotinin, 10 \( \mu \text{mol/L} \) leupeptin, 10 \( \mu \text{mol/L} \) NaF, 1 mmol/L Na\(_3\)VO\(_4\), and 1 mmol/L phenylmethylsulfonyl fluoride] and 70 \( \mu \text{g} \) protein/well was run (PAGE), blotted, and probed for HIF-1\(a\) and \( \alpha \)-actin. Proteins were visualized with enhanced chemiluminescence (Amersham).

**Pharmacokinetics**

Pharmacokinetic variables were determined after oral administration. Compounds were formulated in 40% hydroxypropyl-\(\gamma\)-cyclodextrin/33.3% DMSO (Wacker Chemical) in water for CD1 mice or were formulated as a suspension (on behalf of EntreMed) for use in male beagle dogs. The liquid solution and suspension formulations were shown to give equivalent plasma exposure for 2ME2 and ENMD-1198 in mice (data not shown). Animals received a single oral dose of compound at 15 mg/kg (mice) or 18 mg/kg (dogs). Following administration of the compounds to mice, blood was collected at predetermined time points by cardiac puncture into tubes with anticoagulant following \( \text{CO}_2 \) inhalation. Samples from three mice per group were pooled for analysis. Blood from dogs (two dogs per compound) was obtained from jugular vein bleeds and collected into tubes with anticoagulant. Blood was collected from animals at nine time points from 15 min to 24 h following dosing. Plasma was separated by centrifugation, plasma proteins were precipitated by addition of organic solvent (methanol or acetonitrile), and samples of all animals were analyzed by high-performance liquid chromatography interfaced with a tandem mass spectrometer operating in multiple reaction mode (limit of detection is 1 ng/mL). The amount of parent compound in each sample was determined by comparison to a 10-point standard curve, and the pharmacokinetic variables were determined using a non-compartmental model (WinNonlin).

**Mouse Tumor Models**

**Compound Dosing.** Dilutions of compounds formulated as a suspension were prepared using sterile water to deliver 100 or 200 mg/kg as an oral dose to tumor bearing mice. LLC metastatic tumor bearing mice were treated at 100 and 200 mg/kg, and MDA-MB-231 orthotopic breast cancer tumor bearing mice were treated with 200 mg/kg. Vehicle doses were made up with an equivalent amount of excipients in sterile water. Mice were dosed orally on a daily basis with the schedules described below.

**Metastatic LLC Model.** Male C57BL/6j mice (6 weeks old; Jackson Laboratories) were provided water and an autoclaved standard rodent diet consisting of 20% protein, 9% fat, 4% fiber, 6.5% ash, 13% moisture, and 2.5% minerals (given \( \text{ad libitum} \)). Exponentially growing LLC cells were harvested by trypsinization. The cells were pelleted, resuspended in PBS, and diluted with ice cold PBS to a final concentration of 1.25 \( \times 10^6 \)/mL. Mice received 0.2 mL (2.5 \( \times 10^6 \) cells) via tail vein on day 0. Oral treatment with analogues was initiated on day 3 via a 20-gauge gavage needle with a dose volume of 10 mL/kg. The treatment groups (\( n = 10 \) mice per group) were control (diluent alone), ENMD-1198 ENMD-1200, and ENMD-1237. Cyclophosphamide treatment was included as a positive control.
control; animals were treated with 150 mg/kg q.o.d. × 3 i.p. All animals were weighed twice weekly and observed daily for clinical signs of adverse, drug-related side effects. The median survival time (MST) of each treatment group was determined.

**Orthotopic MDA-MB-231 Model.** CB.17 SCID mice (female, 10 weeks old; Jackson Laboratories) were provided water and an autoclaved standard rodent diet consisting of 20% protein, 9% fat, 4% fiber, 65% ash, 13% moisture, and 2.5% minerals (given ad libitum). Exponentially growing MDA-MB-231 cells (5.0 × 10⁶) were injected s.c. in 0.1 mL into the right mammary fat pad on day 0. Daily oral administration of compounds formulated as a suspension was initiated with six mice per group when the tumors were palpable, starting at day 12. The study was terminated at day 33 due to the size of the control (vehicle-treated) tumors.

**Statistical Analysis**
Results from the LLC metastases model were analyzed for statistical significance using the Cox proportional hazards model (P < 0.05). Results from the MDA-MB-231 orthotopic model were analyzed for statistical significance using ANOVA, Bonferroni multiple comparisons (P < 0.05).

**Results**

**Antiproliferative Activities of Compounds In vitro**
ENMD-1198, ENMD-1200, and ENMD-1237 (Fig. 1B) were evaluated for in vitro antiproliferative activity in tumor cell lines and in human umbilical vein endothelial cells to determine antitumor and angiogenic activities. As shown in Table 1A and B, the three compounds had activity against a variety of tumor and endothelial cell types and, like 2ME2, are not substrates for multidrug resistance pumps. Several cell lines, such as HT-29, are resistant to the antiproliferative activity of 2ME2, with an IC₅₀ value of 82.5 μmol/L, yet HT-29 cells were sensitive to ENMD-1198, ENMD-1200, and ENMD-1237 (IC₅₀ values of 0.44, 0.78, and 3.64 μmol/L, respectively). Overall, ENMD-1198 and ENMD-1200 were more potent than 2ME2 in all the cell lines examined with median GI₅₀ values in the National Cancer Institute cell screen of 0.10 and 0.34 μmol/L, respectively, compared with 2.46 μmol/L for 2ME2 (Table 1B). Except for its greater potency with HT-29 cells, ENMD-1237 generally had activity similar to that of 2ME2, including the National Cancer Institute screen, where it yielded a GI₅₀ of 2.22 μmol/L.

**Treatment of cells with 2ME2 results in G₂-M cell cycle arrest followed by apoptosis (4, 5). To determine if the compounds showed similar activities and kinetics, MDA-MB-231 cells were treated with the compounds at two times their IC₅₀ values for 4, 8, 16, or 24 h, fixed, and stained with propidium iodide. Cell cycle analysis was done using a Guava cell analyzer. As shown in Table 1C, 2ME2, ENMD-1198, ENMD-1200, and ENMD-1237 all induce G₂-M arrest after 4 to 16 h of treatment and apoptosis after 16 to 24 h of treatment. These data suggest that all the compounds have a similar antiproliferative and apoptotic mechanism of action.

**Inhibition of Tubulin Assembly and Colchicine Binding**
Microtubule destabilization by 2ME2 has been shown to occur through binding at the colchicine binding site (4, 25). To evaluate whether chemical modifications at positions 3 and 17 of the analogues affected binding of the analogues to tubulin, we measured inhibition of assembly of purified brain tubulin by 2ME2, ENMD-1198, ENMD-1200, and ENMD-1237 and inhibition of labeled colchicine binding to tubulin in the presence of each molecule (Table 2). Consistent with their potency on cellular proliferation (Table 1), ENMD-1198 and ENMD-1200 inhibited microtubule polymerization most effectively (IC₅₀ values of 2.6 and 2.5 μmol/L, respectively), whereas ENMD-1237 had an IC₅₀ value of 3.6 μmol/L. All the analogues showed lower IC₅₀ values than 2ME2 (5.4 μmol/L) and inhibited colchicine binding to tubulin by ≥50% at 5 μmol/L compared with 17% inhibition by 2ME2. The slightly increased potency of ENMD-1237 in this assay was unexpected (Table 2), because it showed comparable to slightly less activity compared with 2ME2 in inhibiting cell proliferation (Table 1). These results are consistent with ENMD-1198, ENMD-1200, and ENMD-1237 binding to tubulin at the colchicine binding site and thus resulting in inhibition of microtubule polymerization, G₂-M cell cycle arrest, and induction of apoptosis.

**Disruption of Microtubules in Cells**
To determine whether the compounds disrupt microtubules in cells, we employed indirect immunofluorescence (Fig. 2A). Disruption of microtubules was compared after an 18-h treatment with 0.5 or 5 μmol/L compound (Fig. 2A, C, E, G, and I or D, F, H, and J, respectively) or untreated controls (Fig. 2A, A and B). Figure 2A illustrates the spindle disruption after exposure of human MDA-MB-231 cells to ENMD-1198 (Fig. 2A, E) and ENMD-1200 (Fig. 2A, G) at 0.5 μmol/L, with little apparent effect on microtubules by ENMD-1237 (Fig. 2A, I) and 2ME2 (Fig. 2A, C) when used at 0.5 μmol/L. At 5 μmol/L, all compounds disrupted microtubules (Fig. 2A, D, F, H, and J), with greater disruption by ENMD-1198 and ENMD-1200.

**Table 2. Inhibition of tubulin polymerization and competition for colchicine binding to tubulin**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Inhibition of polymerization IC₅₀ (μmol/L)</th>
<th>Percent inhibition [³H]colchicine binding</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 μmol/L</td>
<td>50 μmol/L</td>
</tr>
<tr>
<td>2ME2</td>
<td>5.4 ± 0.5</td>
<td>17 ± 7</td>
</tr>
<tr>
<td>ENMD-1198</td>
<td>2.6 ± 0.4</td>
<td>56 ± 2</td>
</tr>
<tr>
<td>ENMD-1200</td>
<td>2.5 ± 0.1</td>
<td>59 ± 5</td>
</tr>
<tr>
<td>ENMD-1237</td>
<td>3.6 ± 0.04</td>
<td>65 ± 3</td>
</tr>
</tbody>
</table>

**NOTE:** The mean IC₅₀ for the inhibition of tubulin polymerization was defined as the compound concentration required to inhibit tubulin assembly by 50% at 20 min. All compounds were examined in at least two independent assays to provide the values as mean ± SD IC₅₀. The mean percent inhibition for colchicine binding was determined using 2ME2 and analogues at 5 and 50 μmol/L with 5 μmol/L [³H]colchicine. ND, not done.
Figure 2. A, indirect fluorescent imaging of disruption of microtubules by compounds. MDA-MB-231 cells were plated onto eight-well glass slides and allowed to attach overnight. Cells were treated with 0.5 μmol/L (C, E, G, and I) or 5 μmol/L (D, F, H, and J) compound for 18 h in fresh medium or with fresh medium alone (untreated, A and B). After fixing in PHEMO, anti-tubulin antibody was applied followed by Alexa 488–conjugated anti-rat antibody (visualized with a FITC filter). Significant disruption of microtubules is apparent in 0.5 μmol/L ENMD-1198-treated and ENMD-1200-treated cells. Magnification, ×300. C and D, 0.5 and 5 μmol/L 2ME2, respectively; E and F, 0.5 and 5 μmol/L ENMD-1198, respectively; G and H, 0.5 and 5 μmol/L ENMD-1200, respectively; I and J, 0.5 and 5 μmol/L ENMD-1237, respectively. B, reduction in HIF-1α protein levels under normoxia and hypoxia. MDA-MB-231 were treated with 0.5 or 5 μmol/L compound overnight and then subjected to hypoxia (1% O2) or continued at normoxia (21% O2) for 4 h. Cells were harvested and cellular lysates were evaluated for HIF-1α protein levels using Western blot analysis.
(Fig. 2A, F and H, respectively). These results are consistent with ENMD-1198 and ENMD-1200 being more potent than 2ME2 and ENMD-1237 at destabilizing microtubules in cells.

**Decrease in HIF-1α Protein Levels**

Mabjeesh et al. showed that 2ME2 reduces HIF-1α protein levels (5). To evaluate whether the analogues affected HIF-1α protein levels, MDA-MB-231 cells treated overnight with 0.5 or 5 μmol/L compound were then subjected to hypoxia (1% O2) or continued at normoxia (21% O2) for 4 h followed by Western blot analysis (Fig. 2B). Under both normoxia and hypoxia, all the compounds decreased HIF-1α protein levels at 5 μmol/L. ENMD-1198 and ENMD-1200 were more potent at inhibiting HIF-1α, exhibiting a marked reduction in the protein level at 0.5 μmol/L.

**Decreased Metabolism**

Initial evaluation for improved pharmacokinetic phase I and/or II metabolic stability was done by assessing the compounds for loss of parent molecule after incubation with rat and human hepatocytes. As shown in Table 3A, following a 2-h incubation with hepatocytes from both species, there was no 2ME2 remaining. In contrast, for ENMD-1198, 67% and 70% of the unmetabolized compound remained in the medium of the rat and human cells, respectively. ENMD-1200 and ENMD-1237 were also metabolically stable, with 87% and 91% remaining for ENMD-1200 and 85% and 98% remaining for ENMD-1237 (rat and human, respectively). These data indicate that these modifications at positions 3 and 17 resulted in molecules with markedly improved metabolic stability in vitro.

**Pharmacokinetic Profiles**

To determine whether the modifications of the hydroxyl moieties at positions 3 and 17 resulted in increased plasma exposure after oral delivery, pharmacokinetic variables of 2ME2, ENMD-1198, ENMD-1200, and ENMD-1237, administered to mice at 15 mg/kg (45 mg/m²) and to dogs at 18 mg/kg (360 mg/m²), were calculated using a non-compartmental model. Pharmacokinetic analysis showed that the analogues have improved pharmacokinetic profiles compared with 2ME2 in both species (Table 3B). In mice, the maximal plasma levels (Cmax) of the analogues ranged from a 1.1-fold for ENMD-1200 to a 4.9-fold increase for ENMD-1198 compared with 2ME2. All three analogues showed >15-fold increase in plasma concentration-time curves (AUC, 18- to 24-fold higher than with 2ME2). In dogs, the analogues showed >50-fold increase in both maximal plasma levels (Cmax, 86- to 170-fold increase) and plasma concentration-time curves (AUC, 78- to 167-fold increase) relative to 2ME2. The terminal half-life (t1/2) of the analogues in mice was improved over 2ME2, but in dogs oral dosing of 2ME2 resulted in a longer half-life. Terminal half-life of 2ME2 is increased in dogs due to intrahepatic circulation.3 Comparing plasma levels of the analogues in mice, dogs (Table 3B), and rats (data not shown), the three analogues show fairly similar pharmacokinetic profiles when corrected for dose.

**In vivo Antitumor Activity**

Based on improved antiproliferative activity in vitro and pharmacokinetic profile of the analogues, all three lead molecules were assessed for antitumor activity in vivo. Two different tumor models, a LLC metastasis survival model and an orthotopic MDA-MB-231 xenograft model, were...
used to evaluate activity after oral administration in comparison with the approved agent cyclophosphamide. For the survival study, LLC cells were injected in the tail vain of C57BL/6J mice, and three days later, mice were treated orally with either 100 or 200 mg/kg compound daily. All analogues were well tolerated and did not cause any weight loss (data not shown). Cyclophosphamide was administered at a maximum tolerated dose (150 mg/kg q.o.d. × 3 i.p.). The MST of the vehicle control-treated animals was 23 days (Fig. 3A). Treatment with ENMD-1198 lead to a statistically significant increase in MST of 53 days (P < 0.05 versus vehicle control) and was comparable with the therapeutic activity of cyclophosphamide (MST, 51 days). Mice treated with 200 mg/kg ENMD-1237 had a MST of 39 days (P < 0.05 versus vehicle control). Surprisingly, despite showing comparable in vitro activity and pharmacokinetic profile as ENMD-1198, the MST of mice treated with ENMD-1200 was statistically indistinguishable from vehicle control-treated mice. At lower doses (100 mg/kg), the MST of mice treated with ENMD-1198 or ENMD-1237 was similar (MST, 33 and 32 days, respectively) and statistically significant compared with vehicle-treated mice (P < 0.05).

Orally administered compounds were also evaluated for antitumor activity in a human mammary carcinoma model (Fig. 3B). MDA-MB-231 cells were orthotopically implanted and treatment was initiated when tumors reached ~100 mm³ (day 12). Mice were treated daily with 200 mg/kg of each analogue until the control tumors reached ~2,500 mm³ (day 33). All three analogues showed a reduction in tumor growth that was statistically significant compared with vehicle control. ENMD-1198 was the most potent analogue, resulting in a 77% reduction in tumor volume. ENMD-1200 and ENMD-1237 also showed antitumor activity, with 49% and 63% reductions, respectively. Therefore, using two different tumor models and assessing either survival or tumor growth inhibition, ENMD-1198 showed the most potent antitumor activity of the three analogues.

**Discussion**

Microtubules play a critical role in many cellular processes, including maintenance of cell shape, intracellular transport, and segregation of chromosomes during cell division by mitotic spindle formation. MTA have proven to be

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**Figure 3.** A, increased median survival time in mice with metastatic LLC tumors by orally administered analogue ENMD-1198 and ENMD-1237. In vivo activity using LLC metastasis survival model. Male C57BL/6 J mice (10 per group) inoculated with LLC cells were treated every day orally with compound starting on day 3. A, graphical depiction of survival of animals treated orally with vehicle (●) or 200 mg/kg ENMD-1198 (○), ENMD-1200 (▲), or ENMD-1237 (○). MST shown in days, with fold increases in MST compared with diluent control indicated in parentheses. Cyclophosphamide (CTX) (●) was administered i.p. (150 mg/kg q.o.d. × 3). *P < 0.05, MST was significantly increased over control vehicle-treated animals as determined by the Cox proportional hazards model (JMP software). B, final mean ± SE tumor volumes (mm³; six mice per group) measured on the day of sacrifice (day 33). Treatment of palpable orthotopic MDA-MB-231 breast tumors began on day 12. *P < 0.05, final tumor volume of treated group was significantly different from vehicle control group as determined by ANOVA using Bonferroni multiple comparisons test (GraphPad InStat software).
successful in the clinic in the treatment of a variety of solid and hematologic cancers (1). However, one of the limitations in the treatment of cancer is the development of resistance to the therapeutic agent. Resistance to taxanes and Vinca alkaloids has been associated with transport by the membrane transporter P-glycoprotein, the product of the multidrug resistance gene MDR1 (1, 2, 26). Also, dosing of MTA is often limited by severe toxicity. New tubulin-binding agents with improved tolerability and that are not substrates for multidrug-resistant pumps have the potential to show efficacy against late-stage resistant tumors (1, 3).

2ME2 is a natural product (metabolite of estradiol) that destabilizes microtubules in vitro and in vivo at pharmacologic doses (5). Initial assessment of 2ME2 in human clinical trials in metastatic breast cancer, prostate cancer, and various solid tumors showed 2ME2 is well tolerated with no major toxicities. However, the metabolism of 2ME2 limits its bioavailability (11, 12, 14, 15).

2ME2 is an attractive lead structure for the generation of a new series of MTA. Several groups have reported 2ME2 analoguing approaches, with the aim of increasing potency against tubulin and antiproliferative activity against tumor cells. The majority of these analogues have focused on modifying the A and D rings, and several analogues have been reported which have improved potency in vitro, including 2-ethoxyestradiol, 2-methoxymethylestradiol, and 14-dehydro-2ME2 (23, 27–31). However, these molecules all retain the hydroxyl groups and thus would be subject to similar metabolism to 2ME2 in vivo. Indeed, we have found that despite an in vitro 10-fold increase in antiproliferative activity for 2-ethoxyestradiol, its potency in vivo was equivalent to that of 2ME2.3

Clinical pharmacokinetic studies with 2ME2 suggest that a strategy directed at altering metabolism by modifying the two hydroxyl groups might be an attractive approach to improving potency and decreasing metabolism of new analogues. In several large series of analogues, we identified few substituents that were both tolerated for in vitro antiproliferative activity and resulted in improved pharmacokinetic variables. The 3-carboxamide resulted in improved antiproliferative activity and plasma drug levels5 and was selected as the lead substituent for this series of molecules. In addition to our work, Ireson et al. have published improved in vitro and in vivo potency and pharmacokinetic variables of 2-methoxymethylestradiol-3,17-O, O-bis-sulfamate (32). At position 17, we found a wider range of chemical substitutents was tolerated. The 16,17-olefin, 17-methylene, and 17-deoxy groups were selected for further study. Details of the synthesis and structure-activity relationships of these analogue series will be published separately.

Substituent changes at positions 3 and 17 resulted in decreased metabolism by hepatocytes in vitro and in increased peak plasma drug levels and AUC in vivo, providing increased exposure in animals when compared with 2ME2 (Table 3A and B). Treatment with ENMD-1198 and ENMD-1237 resulted in statistically significant increases in MST and tumor growth inhibition (Fig. 3). Despite having similar antiproliferative activity as ENMD-1198 (Table 1), ENMD-1200 did not show as potent antitumor activity in the metastatic LLC or the orthotopic breast carcinoma tumor models (Fig. 3). Comparison of pharmacokinetic variables for the analogues indicates that, in mice, the Cmax levels of ENMD-1200 were 1.5 to 5 times lower than ENMD-1237 and ENMD-1198, although the AUC were comparable. Intratumoral drug levels and tissue distribution have not been determined and may account for the differences in activity observed between the analogues.

The three lead analogues and 2ME2 all have comparable IC50 values for MDR-overexpressing cell lines compared with parental lines. These data are consistent with these compounds not being substrates of the P-glycoprotein membrane transporter. Recent reports also confirm that 2ME2 is effective in inhibiting cancer cell lines resistant to other MTA (5, 33, 34). Similar results have been obtained with ENMD-1198 (data not shown). Escuin et al. have reported preliminary data indicating that cell lines, which have been made resistant to 2ME2 and contain mutations in the colchicine binding site of tubulin, are only modestly resistant to ENMD-1198 (33). It is also interesting that some differences in cell line sensitivities have been observed in comparing the activity of ENMD-1198 and 2ME2. A few cell lines showed sensitivity to ENMD-1198 but not to 2ME2 (e.g., HT-29 colon carcinoma cells); the converse was also observed in which some cell lines were sensitive to 2ME2 but not to ENMD-1198 (data not shown). Studies are ongoing to assess similarities and differences in the mechanisms of action of 2ME2 and ENMD-1198 and to determine whether the degree of sensitivity to these agents is related to known resistance mechanisms. Although there are some cell type–specific differences observed in compound sensitivities, all three analogues have a similar mechanism of action as 2ME2 in that they bind the colchicine binding site of tubulin, destabilize microtubules, induce G2-M cell cycle arrest and apoptosis, and decrease HIF-1α protein levels.

2ME2 is unique among the MTA as it has a benign toxicity profile and does not result in myelosuppression or neurotoxicity even in patients who have been treated daily with 2ME2 for >5 years (11, 12, 14, 15). A new formulation of 2ME2 with improved bioavailability (Panzem® Nano-Crystal® dispersion) has entered phase I and II clinical trials (16, 18). The dose-limiting toxicity reported for this formulation is fatigue with occasional transient elevations in liver enzymes. Clinical benefit has been reported (16, 18, 35, 36). In contrast, preclinical evaluation indicates that ENMD-1198 has dose-limiting toxicities of myelosuppression and gastrointestinal effects (57). Although no evidence of neurotoxicity has been observed to date, larger clinical trials are necessary to evaluate this observation. It is interesting to note that another colchicine-site MTA, MKC-1 (previously Ro 31-7453), has been administered to

3 In preparation.
>300 cancer patients and exhibits a dose-limiting toxicity of myelosuppression but similarly has no reported neurotoxicity, suggesting that activity might be able to be separated from this toxicity (38, 39).

In summary, by altering the substituents of 2ME2 at positions 3 and 17, we have created novel molecules with increased in vitro antitubulin, antiproliferative, and HIF-1α activities, increased metabolic stability, and improved pharmacokinetic profiles. The significant antitumor activity shown in vivo, particularly by ENMD-1198, resulted in enhanced survival and strong tumor growth inhibition in metastatic and primary tumor models. The broad range of antiproliferative activity shown by analogue ENMD-1198, combined with its oral bioavailability and preclinical antitumor activity at well tolerated doses, support the selection of this molecule for further studies in humans. ENMD-1198 is currently in phase I oncology clinical trials for patients with refractory tumors.

Disclosure of Potential Conflicts of Interest


References

Significant antitumor activity in vivo following treatment with the microtubule agent ENMD-1198

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