Chemical Therapeutics

A Second-Generation 2-Methoxyestradiol Prodrug Is Effective against Barrett's Adenocarcinoma in a Mouse Xenograft Model

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Abstract

2-Methoxyestradiol (2-ME2) is an endogenous metabolite of estradiol. In preclinical models, 2-ME2 is effective against different types of tumors. Unfortunately, only low systemic concentrations of 2-ME2 can be achieved following oral administration, even after very high doses are administered to patients. In an effort to solve this problem, we have now synthesized and tested a new prodrug of 2-ME2 that is water-soluble due to a bioreversible hydrophilic group added at the 3-position and that more effectively resists metabolic inactivation due to an ester moiety added to mask the 17-position alcohol. We are reporting here for the first time that this double prodrug of 2-ME2 is effective as an antiproliferative and anticancer agent for both in vitro and in vivo studies against Barrett esophageal adenocarcinoma (BEAC) and provided greater potency than 2-ME2 in inhibiting the growth of BEAC xenografts. Finally, studies indicate that, like 2-ME2, the 2-ME2-PD1 exhibits anticancer effect through possible disruption of microtubule network. Mol Cancer Ther; 12(3); 255–63. ©2012 AACR.

Introduction

2-Methoxyestradiol (2-ME2) is an endogenous metabolite of 17β-estradiol (E2), which is generated from a 2-step activation process involving sequential hydroxylation at the 2C position of 17β-estradiol by cytochrome P450 enzymes followed by methylation at the same position by catechol-O-methyl transferase (COMT; refs. 1, 2). Our laboratory has studied the anticancer activities of 2-ME2 for many years (3–6). In addition, multiple in vitro and in vivo studies have documented that 2-ME2 is effective against many different types of tumors by virtue of its potent antiproliferative, antiangiogenesis, and proapoptotic activities (7–9).

Despite the great promise and broad anticancer properties shown for exogenous 2-ME2, clinical application is limited because the in vivo effect of 2-ME2 is inconsistent and weak due to poor bioavailability of the 2-ME2. The published literature identifies very high patient-to-patient variability in systemic exposure following oral administration. In all cases, there is low serum bioavailability (approximately 1%–2%) and a limited temporal window with measurable serum levels (1, 10–12). Thus, high oral doses of 2-ME2 that present dosing challenges would be necessary to maintain therapeutic level of the drug in the plasma for effective lengths of time.

The major barriers to oral delivery of 2-ME2 to systemic circulation include, but are not limited to, formulation, solubility, permeability, transporter effects, and first-pass metabolism (1). Prodrug approaches have been used with varying degrees of success to overcome all these barriers (13–15). Thus, designing a prodrug of 2-ME2 would be one promising approach to solving the problem of oral delivery. A prodrug is a chemically modified drug designed to be endogenously converted to the active drug. Unfortunately, prior studies devoted to the design and testing of 2-ME2 prodrugs have not produced a successful chemotherapeutic agent (1).

Building on the successes and limitations noted for the first generation of 2-ME2 analogues, we devised a strategy to create improved 2-ME2 prodrugs that exhibit more predictable pharmacokinetics. In summary, we describe
the synthesis of a novel prodrug that, by virtue of its initial assessments, promises to be an effective oral anticancer agent against esophageal cancer.

Materials and Methods

Cell line

OE33 cells, was purchased from American Type Culture Collection (ATCC). OE33 cells used in this study were from fifth through tenth passages and were routinely maintained in Dulbecco’s Modified Eagle’s Medium (Sigma) supplemented with 10% FBS (Hyclone) and antibiotics (Sigma), penicillin (100 U/mL), and streptomycin (100 μg/mL). The cell line authentication and the status of mycoplasma were carefully determined before and after carrying out the experiments needed for these studies. In these studies, we used only one cell line as widely used Barrett esophageal adenocarcinoma (BEAC) cell lines (i.e., BIC-1, SEG-1, and TE-7) are in serious identity crisis (16, 17). FLO-1 cell line, which has been used for various studies as evidenced by citations in PubMed and has been authenticated with the parental tumor, is unavailable now in ATCC.

Reagents

The 2-ME2 was purchased from Sigma, and 20 μmol/L stock solutions of 2-ME2 were prepared in absolute ethyl alcohol and stored at –20°C. All other chemical were obtained either from Sigma or Fisher Scientific (Fisher Scientific Company). All newly synthesized compounds were characterized by using analytic tools.

Animals

Fasted Sprague Dawley rats (male, 250–300 mg) were purchased from Charles River Laboratories. Six- to 8-week-old athymic male athymic nude mice (BALB/c- nu/nu) were obtained from Charles River Laboratories. All animals were maintained in a sterile environment and daily 12-hour light/dark cycle. All the animals were maintained according to standard guidelines of American Association for the Accreditation of Laboratory Animal Care with the approval of the Institutional Animal Care and Use Committee of the Kansas City VA Medical Center (Kansas City, MO) and the University of Kansas (Lawrence, KS).

Rationale for the design of the 2-ME2 prodrug

2-ME2 is a poorly soluble compound with a predicted aqueous solubility of 4.8 μg/mL (calculated using the Advanced Chemistry Development Software V8.14 for Solaris). 2-ME2 possesses poor aqueous solubility over the complete pH profile of the gastrointestinal tract. As bioavailability has been increased through recent developments in nanocrystal formulation (10-K SEC Filing, filed by Entremed Inc. on 3/6/2008), there is evidence

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**Figure 1.** Sequential events of synthesis of the 2-ME2 prodrug, 2-ME2-PD1, from 2-methoxyestradiol. The first step in the synthesis of 2-ME2-PD1 is the selective formation of the methylthiomethyl ether intermediate at the phenolic hydroxyl of 2-ME2 (selectively favored over the secondary alcohol on the opposite side of the molecule). This step was followed by acylation of the secondary alcohol (to protect and hinder metabolism at C-17 position). The acetylated product was purified by column chromatography and used in the formation of the protected phosphate ester. Final removal of the dibenzyl groups on the phosphate ester (C-3 position) and formation of the sodium salt gives the desired prodrug structure.
suggesting that a more soluble form of 2-ME2 would help increase its absolute bioavailability (1, 11). Thus, to overcome the unpredictable pharmacokinetics of the parent 2-ME2 compound, the synthetic efforts have been focused on (i) increasing the aqueous solubility/dissolution rate through the addition of a bioreversible hydrophilic group at the 3-position and (ii) altering drug metabolism by masking the 17-position through covalent addition of an ester moiety (Fig. 1).

**Synthesis of the 2-ME2 prodrug (2-ME2-PD1)**

The synthesis of the main compound, 2-ME2-PD1—disodium ((6R,9S,13S,14S,17S)-17-acetoxy-2-methoxy-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[a]phenanthren-3-yl)oxy)methyl phosphate (C_{22}H_{29}Na_{2}O_{8}P)—is as follows (Fig. 1).

**Step 1.** About 157 mg of OAc MTM 2-ME2 (0.39 mmol) was dissolved in 20 mL of dry dimethylformamide. Sodium hydride was added. The mixture was stirred at room temperature. After 1 hour, the reaction was stirred in vacuo. The resulting solid was dissolved in a 1:2 v/v mixture of ethyl acetate and hexanes. The major product was isolated with elution on a silica gel column with the same solvents (R_{f} = 0.45) to provide 135 mg (86% yield).

**Step 2.** About 208 mg of MTM 2-ME2 (0.57 mmol) and 126 mg acetic anhydride (1.23 mmol, 3 eqv.) were dissolved in 10-mL dry pyridine at 0°C. The reaction was stirred overnight and allowed to come to room temperature. The solvent was removed in vacuo and the resulting solids dissolved in ethyl acetate. The organic layer was washed with water and then filtered through silica gel. The ethyl acetate was removed in vacuo. The solids were dissolved in a 1:2 v/v mixture of ethyl acetate and hexanes and the major product isolated with elution on a silica gel column with the same solvents (R_{f} = 0.81) to provide 157 mg (86% yield). The product was recrystallized from a 1:2 v/v mixture of ethyl acetate and hexanes to provide 120 mg (86% yield).

**Step 3.** About 157 mg of OAc MTM 2-ME2 (0.39 mmol), 325 mg dibenzyl hydrogen phosphate (1.16 mmol, 3 eqv.), and 320 mg N-iodosuccinimide (1.42 mmol, 3.6 eqv.) were dissolved in 5 mL of dry tetrahydrofuran at room temperature. After 1 hour, the reaction was stirred and the solvent was removed in vacuo. The resulting solid was dissolved in a 1:2 v/v mixture of ethyl acetate and hexanes, and the major product isolated with elution on a silica gel column with gradient elution (1:2 v/v ethyl acetate:hexanes to 1:1 v/v ethyl acetate:hexanes; R_{f} = 0.62) to provide 120 mg (86% yield).

**Pharmacokinetic analysis of 2-ME2-PD1 and 2-ME2**

2-ME2-PD1 and 2-ME2 were dissolved independently in 0.2 mol/L hydroxypropyl-β-cyclodextrin (Sigma-Aldrich) or 0.1 mol/L Captisol (Ligand Pharmaceuticals, Inc) at concentrations between 10 and 21 mg/mL. The solutions were sterile-filtered through 0.2-μm filters before use. A cannulated rat model was used to study the intravenous and oral absorption of 2-ME2 and 2-ME2-PD1. Sprague Dawley rats were implanted with carotid artery and jugular and/or femoral vein catheters. These studies were automated with the animals connected to the Culex Automated Pharmacology System allowing for direct comparison of pharmacokinetic behavior between the orally and intravenously administered compounds in the same animal. Following surgery, the animals were connected to the Culex and allowed to recover and acclimate.

Oral doses were given via gavage to the animal under light anesthesia. Blood was sampled at times ranging from 5 to 1,440 minutes into heparinized vials containing a cocktail of phosphatases and esterase inhibitors stored on the chilled fraction collector and remained there until sampling was complete. Blood samples were processed to plasma via centrifugation and stored at −80 °C until analysis for 2-ME2-PD1, 2-ME2, and associated metabolites by liquid chromatography/tandem mass spectrometry (LC-MS/MS). Bioanalytic methods for the 2-ME2-PD1 and 2-ME2 analysis were modified from those of Lakhani and colleagues (18). Quantitation was made relative to deuterated internal standards. Pharmacokinetic (PK) analysis of the resulting plasma concentration time data was conducted using PK Solutions software (Summit PK).

**In vitro cell proliferation assay**

The antiproliferative effect of 2-ME2 and 2-ME2-PD1 on OE33 cells was evaluated using crystal violet cell proliferation assay according to previous method (19). Eight duplicate wells were used for each determination. Inhibition of cell proliferation was calculated as a percentage using following equation:
% of inhibition = (1 – OD of the treated sample/OD of the vehicle-treated sample) × 100

**Induction and treatment of tumor xenograft and sample collection**

OE33 tumor xenografts were generated essentially as previously described (20, 21). Briefly, semiconfluent OE33 cells (2.5 × 10⁶) were resuspended in Matrigel and were injected s.c. into the right hind leg of each mouse for the development of the tumor. The animals were distributed into 3 groups (5 mice/group). After detection of palpable tumor, mice were given daily equivalent doses of 2-ME2 or 2-ME2-PD1 (75 mg/kg/d) by orogastric feeding or vehicle (control). 2-ME2-PD1 was dissolved in saline or 2-ME2 (75 mg/kg/d) by orogastric feeding or tumor, mice were given daily equivalent doses of 2-ME2 into 3 groups (5 mice/group). After detection of palpable development of the tumor. The animals were distributed in statistically significant.

**Student 2-tailed**

between groups were determined by using the paired **t** test. A value of **P** < 0.05 was considered statistically significant.

**Immunofluorescence**

The immunofluorescence procedure was the same as previously described (21, 22). Briefly, for in vitro analysis, OE33 cells were fixed with acetone and permeabilized with 0.1% Triton X-100. For in vivo analysis, tumor xenograft paraffin-embedded tissue sections were deparaffinized and then rinsed in distilled water. Nonspecific binding sites were blocked by Super Block Blocking Buffers (Thermo) followed by incubation with rabbit monoclonal anti-α-Tubulin (Alexa Fluor 555 Conjugate) and counterstained with 4',6-diamidino-2-phenylindole (DAPI). Images were captured by a Nikon Eclipse Ti-U fluorescence microscope.

**Statistical analysis**

Analyses of data were achieved with GraphPad Prism version 4.00 (GraphPad Software). All data are expressed as the mean ± SD. Statistically significant differences between groups were determined by using the paired Student 2-tailed **t** test. A value of **P** < 0.05 was considered statistically significant.

**Results**

**Pharmacokinetics of 2-ME2 prodrug (2-ME2-PD1) relative to 2-ME2**

2-ME2-PD1 was synthesized in 28% overall yield, starting from 2-ME2 as precursor. Unlike 2-ME2, 2-ME2-PD1 readily dissolves in sterile water for easy use and rapid preparation of doses. Validation studies with the phosphatase and esterase inhibitors showed the stability of 2-ME2-PD1 and its ester metabolite in whole blood following sampling. There were no observable effects in the animals following dosing of 2-ME2-PD1 and 2-ME2 in the pharmacokinetic studies. Nonparametric analysis of the resulting plasma concentration–time data was conducted, and the pharmacokinetic parameters were derived following the standard methods (Fig. 2 and Supplementary Table S1 and Table 1; ref. 23). For oral doses of 2-ME2, no compound could be detected in plasma at any time point studied using limit of quantification (LOQ: < 2 ng/mL) showing again the limited bioavailability of oral 2-ME2. Intravenous 2-ME2 dosing does produce measurable drug levels in plasma, but these levels are short-lived because of presumed rapid clearance (Table 1). Our data suggest that the strategy used to develop 2-ME2-PD1 does deliver meaningful levels of 2-ME2 to the systemic circulation for extended periods of time following either oral or intravenous administration. The studies show that therapeutic levels of 2-ME2 are sustained for at least 240 minutes, and significant amounts are present for as long as 480 minutes for the higher doses (31.5 mg/kg of orally administered 2-ME2-PD1; Fig. 2). This suggests, in these limited studies, that the oral bioavailability of 2-ME2-PD1 appears to be dose-dependent. No analyzed sample displayed detectable levels of 2-ME2 when plasma was analyzed at times greater than 480 minutes.

These experiments show that oral 2-ME2-PD1 administration results in a higher systemic exposure relative to 2-ME2. The absolute bioavailability of orally administered 2-ME2-PD1 relative to intravenous administration of 2-ME2 is in the 4% to 5% range. A plasma half-life of 262 minutes is obtained for intravenous 2-ME2-PD1 as opposed to 59 minutes for a comparable intravenous dose of 2-ME2. Orally, 2-ME2-PD1 produces far superior results than 2-ME2 and yields a 2-ME2 plasma half-life of 411 to 807 minutes, which is a marked improvement over what can be obtained with 2-ME2 itself. These data suggest that more detailed and extensive studies should
be conducted to further establish our proof of concept for this new prodrug.

The effect of 2-ME2-PD1 on viability of OE33 cells relative to 2-ME2

Previously, we showed that 2-ME2 dose dependently inhibits the BEAC cell proliferation and the 50% inhibitory concentration (IC\textsubscript{50}) was about 5 \(\mu\text{mol/L}\) of 2-ME2 (21). In this study, we sought to determine whether 2-ME2-PD1 exhibits similar effect on the viability of OE33 cells. To do so, OE33 cells were treated with either 2-ME2-PD1 or 2-ME2 at various concentrations for 48 hours followed by crystal violet dye colorimetric assay for the viability of OE33 cells. As shown in Fig. 3, a dose-dependent inhibition of OE33 proliferation was observed when the cells were exposed to 2-ME2 or 2-ME2-PD1, but 2-ME2 is much more potent than the prodrug. The IC\textsubscript{50} for 2-ME2 was found to be approximately 6 \(\mu\text{mol/L}\) after 48 hours of incubation, whereas for the prodrug, the IC\textsubscript{50} was obtained at approximately 17 \(\mu\text{mol/L}\), indicating that the prodrug provides significantly less in vitro antiproliferative effect on OE33 cells than the parent 2-ME2.

Alteration in cellular morphology of OE33 cells by 2-ME2 and 2-ME2-PD1 treatment

Morphologic alterations, such as vacuolation and atrophy due to membrane blebbing, and cytoplasmic shrinkage are the common cytotoxic effects of an anticancer drug (24, 25). We therefore investigated whether 2-ME2 and 2-ME2-PD1 are able to alter the cellular morphology of OE33 cells. To test this, the phase-contrast images of the treated and untreated cells were studied (Fig. 4A–D). Untreated OE33 cells showed that cells were closely arranged with uniform size, as well as good viability with retained shape, morphology, and refractivity after 48 hours of culture (Fig. 4A), whereas aberrations in the cellular morphology were observed in both 2-ME2- and 2-ME2-PD1–treated cells in a dose-dependent fashion. In the presence of 2.5 \(\mu\text{mol/L}\) of 2-ME2-PD1, only mild to no alterations in the cellular morphology were observed (Fig. 4B), but when exposed to 5 \(\mu\text{mol/L}\) of 2-ME2-PD1, the cells displayed atrophy (Fig. 4C). In contrast, the effect of each concentration of 2-ME2 (i.e., 2.5 or 5 \(\mu\text{mol/L}\)) on cellular morphology was much more drastic than 2-ME2-PD1–treated (Fig. 4D).

Disruption of the microtubule network in OE33 cells by 2-ME2 and 2-ME2-PD1 treatment

As antimitotic and antiproliferative action of 2-ME2 is mediated via microtubule disruption (26), we sought to determine whether the cellular microtubule network also acts as a target for the 2-ME2-PD1 actions. To do so, immunofluorescence against \(\alpha\)-tubulin in 2-ME2–treated, 2-ME2-PD1—treated, or untreated OE33 cells was conducted to determine whether the prodrug is mimicking

<table>
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<tr>
<th>Route</th>
<th>2-ME2 Dose, mg/kg</th>
<th>2-ME2 Corrected dose, mg/kg</th>
<th>2-ME2 AUCn-480, ng min mL(^{-1})</th>
<th>2-ME2 C\textsubscript{max}, ng/mL</th>
<th>2-ME2 T\textsubscript{1/2}, min</th>
<th>2-ME2 Bioavailability, %</th>
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<tr>
<td>Intravenous</td>
<td>5.21</td>
<td>5.21</td>
<td>19,253</td>
<td>1,221</td>
<td>59</td>
<td>100</td>
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<tr>
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<td>6.06</td>
<td>4,967</td>
<td>262</td>
<td>121</td>
<td>21.8</td>
</tr>
<tr>
<td>Oral</td>
<td>20</td>
<td>12.13</td>
<td>2,006</td>
<td>807</td>
<td>807</td>
<td>4.4</td>
</tr>
<tr>
<td>Oral</td>
<td>31.5</td>
<td>19.11</td>
<td>3,516</td>
<td>411</td>
<td>4.9</td>
<td></td>
</tr>
</tbody>
</table>

NOTE: Sprague Dawley rats were implanted with carotid artery and jugular and/or femoral vein catheters. The Culex Automated Pharmacology System–facilitated pharmacokinetic analysis of the resulting plasma concentration–time data was conducted using PK Solutions software.
Abbreviation: AUC, area under the curve.

*Bioavailability values are through 480 minutes.
cells when treated with 2.5 μmol/L 2-ME2. Microtubule disruption was not very prominent in OE33 treated cells, organized microtubules were the function of 2-ME2 under similar in vitro conditions. In untreated OE33 cells, organized microtubules were observed with characteristic fibrous network (Fig. 4E). Microtubule disruption was not very prominent in OE33 cells when treated with 2.5 μmol/L 2-ME2-PD1 for 48 hours or left untreated, and cellular morphologic changes were monitored using a Nikon Eclipse Ti-U phase-contrast microscope (A–D, left). To determine the microtubule disruption by 2-ME2 and 2-ME2-PD1, semi-confluent OE33 cells were exposed to 2-ME2 or 2-ME2-PD1 for 48 hours or left untreated. Treated or untreated cells were fixed and stained with TRITC-conjugated antibody to anti-α-tubulin to visualize microtubules (red) and counterstained with DAPI to visualize nuclei (blue). Images of the cellular microtubules were captured using a Nikon Eclipse Ti-U fluorescence microscope (E–H, right).

Figure 4. The differential effect of 2-ME2 and 2-ME2-PD1 on cellular morphology and microtubule organization in OE33 cells in vitro. To study cellular morphology, semi-confluent OE33 cells were exposed to 2-ME2 or 2-ME2-PD1 for 48 hours or left untreated, and cellular morphologic changes were monitored using a Nikon Eclipse Ti-U phase-contrast microscope (A–D, left). To determine the microtubule disruption by 2-ME2 and 2-ME2-PD1, semi-confluent OE33 cells were exposed to 2-ME2 or 2-ME2-PD1 for 48 hours or left untreated. Treated or untreated cells were fixed and stained with TRITC-conjugated antibody to anti-α-tubulin to visualize microtubules (red) and counterstained with DAPI to visualize nuclei (blue). Images of the cellular microtubules were captured using a Nikon Eclipse Ti-U fluorescence microscope (E–H, right).

The effect of 2-ME2-PD1 on OE33 xenograft tumors relative to 2-ME2

In the next set of experiments, we compared the in vitro effects of 2-ME2-PD1 with 2-ME2 on OE33 xenograft tumors. As illustrated in Fig. 5, our data show that 2-ME2-PD1 strongly and consistently, in a time-dependent fashion, inhibited the growth of established OE33 xenografts in nude mice after 12 days of treatment as compared with the control group as well as 2-ME2-treated group where the effect was inconsistent. The parental 2-ME2 effect is in agreement with our previous work (21). Tumor size increased much more rapidly in the control animals relative to prodrug-treated group. Thus, the studies indicate that the prodrug appeared to provide better antitumor activity than did the parent compound in these experiments.

Because our in vitro studies showed that both 2-ME2 and 2-ME2-PD1 alter cellular morphology and microtubules distribution in OE33 cells, we investigated whether prodrug similarly affects cellular morphology and microtubule networking under in vivo microenvironment. To do so, the distribution of microtubules was characterized in 2-ME2-PD1–treated and untreated tumor xenograft sections by immunofluorescence analysis using anti-α-tubulin antibody. Consistent with in vitro data (Fig. 4), studies show striking alteration in microtubule distribution in conjunction with cellular morphology suggestive of mesenchymal (spindle-shaped)-to-epithelial (cuboidal) transition (MET) in 2-ME2-PD1–treated OE33 tumor xenografts as compared with untreated samples (Fig. 6).

Figure 5. The differential effect of 2-ME2 and 2-ME2-PD1 on OE33 tumor xenografts in nude mice. OE33 cells with Matrigel were injected s.c. into the right hind leg of each athymic nude mice as described in Materials and Methods. After palpable tumors developed, animals were exposed to 2-ME2 or 2-ME2-PD1 or vehicle alone every day for 12 days. The change in tumor volume is plotted for untreated (control), 2-ME2 treated, and 2-ME2-PD1 treated. Bars, SD. *, P < 0.01; **, P < 0.001; #, P < 0.005 versus untreated.
Discussion

The present study effectively formulated a novel prodrug of 2-ME2 (2-ME2-PD1) and tested its antiproliferative and antitumorigenic efficacy in BEAC tumor models. The studies show that unlike parent 2-ME2, the 2-ME2-PD1 is water soluble, and the bioavailability of orally administered 2-ME2-PD1 is significantly higher than intravenous and/or oral administration of 2-ME2 (Fig. 2). Although the 2-ME2-PD1 does not inhibit the in vitro growth of BEAC cells as effectively as parent 2-ME2 does (Fig. 3), the antitumorigenic action of 2-ME2-PD1 on BEAC xenograft is highly and significantly effective than the parent molecule (Fig. 5). Moreover, the antitumorigenic effect 2-ME2-PD1 on BEAC cells, like 2-ME2, is predominantly mediated through altering the microtubule networks as well as morphologic changes from MET version occurs.

Mesenchymal (spindle shaped) Epithelial (cuboidal shaped)

Figure 6. The differential effect of 2-ME2-PD1 on cellular morphology and microtubule organization in OE33 tumor xenograft. 2-ME2-PD1-treated or untreated (control) tumor tissue samples were collected and immunofluorescent staining of microtubule filaments (red) in paraffin-embedded sections of the tumors was conducted using monoclonal anti-α-tubulin antibody.

Esophageal cancer remains a leading cause of cancer mortality worldwide, and in the United States, it is the seventh leading cause of cancer death (27). Thus, there is an urgent need to investigate effective and less toxic chemotherapeutic options to effectively combat this dangerous cancer. In pursuit of this goal, we have studied the antitumor properties of 2-ME2 in our laboratory for many years. We and others have found this compound to be effective against many different types of cancer (3, 4, 21, 28–30). Unfortunately, because of the limited aqueous solubility and less bioavailability, very high oral doses of 2-ME2 are needed to maintain therapeutic plasma levels of drug for an effective length of time, which is a great hindrance to its use as an anticancer drug (1, 10, 31, 32). Thus, designing the proper prodrug to defeat first-pass metabolic inactivation seems to be the best approach in developing a practical therapeutic agent from 2-ME2.

To advance our earlier work focused on BEAC, we undertook the task of designing a better prodrug of 2-ME2 by following a 2-prong strategy directed at increasing aqueous solubility through addition of a bioreversible hydrophilic group at the 3-position and then altering in vivo metabolism by masking the 17-position through the covalent addition of an ester moiety (Fig. 1). The 3-position promoiety is designed to be cleaved at the brush border of the intestinal epithelium providing high local concentrations of the prodrug intermediate for intestinal absorption. On the first pass through the intestinal epithelium and liver, the masked 17-position will undergo de-esterification. Our results show that oral administration of this double 2-ME2 prodrug, 2-ME2-PD1, provides an absolute bioavailability of 4% to 5% (Fig. 2), and this is a significant improvement over what is observed with oral 2-ME2 and the first-generation prodrugs of 2-ME2 (1). The first-generation prodrugs did not include a methylene spacer between the alcohol and the phosphate ester group. The addition of the spacer greatly increases the ability of phosphatases to cleave the phosphate ester en route to the generation of 2-ME2.

It is noteworthy that 2-ME2-PD1 is easily dissolved in sterile water for injection, and this made the prodrug much easier to use for in vitro dosing than 2-ME2, which being nonaqueous is usually dissolved in DMSO. In the in vitro experiments, comparison between the effect of 2-ME2 and the 2-ME2-PD1 on the viability of OE33 cells showed that the IC50 values of the 2-ME2-PD1 were about 3-fold higher than those of 2-ME2 (Fig. 3). Although the in vitro mechanisms of 2-ME2-PD1 are not yet fully known, these results suggest that the active compound, 2-ME2, is being generated at the cellular level. This is not unexpected as alkaline phosphatase is present in cellular membranes and esterases are prevalent intracellularly. However, at the cellular level, we have not yet determined whether through enzyme-mediated hydrolysis the prodrug is being converted to 2-ME2, and if so, it would be helpful to know the actual efficiency at which this conversion occurs.

The results of our in vivo experiments indicate that 2-ME2-PD1 has potent antitumor activity against BEAC xenograft tumor in nude mice over 12 days. An oral dose of 75 mg/kg/d of 2-ME2-PD1 significantly inhibit the growth of OE33-induced tumors in nude mice with reduction in tumor volume of 60% ± 5% relative to the control group and 2-ME2 group as well (Fig. 5). This result is highly significant as the prodrug dose is equivalent to a 45.5 mg/kg/d dose of 2-ME2. More simply stated, the prodrug dosed at 60% the level of 2-ME2 produced significantly higher reduction in tumor volume relative to 2-ME2. Thus, confirming that, as compared to tissue culture conditions, in the enzyme-rich environment of the OE33-tumor xenografted animals, there is more efficient and preferential conversion of 2-ME2-PD1 into systemically bioavailable 2-ME2.

Under in vitro conditions, 2-ME2 is known to inhibit the polymerization of the purified tubulin (26, 33) and impair microtubule dynamics (34) to mediate its tumor-suppressive actions on cancer cells. Here, we report that the antiproliferative effects of 2-ME2-PD1 on OE33 cells mimic those of its parent derivative, 2-ME2, and we provide evidence that these effects are pharmacodynamically
mediated by the 2-ME2-PD1–induced dose-dependent alteration of the microtubule network as well as cellular architecture of these cells (Figs. 4 and 6). Normally, the morphology of OE33 cells is spindle-shaped of mesenchymal type. However, following treatment with parental 2-ME2 or 2-ME2-PD1, the morphology drastically changes from MET architecture (Figs. 4 and 6). Future studies are warranted to study the signaling pathways that are involved in 2-ME2-PD1–mediated MET event.

In summary, 2-ME2-PD1 functions as an effective prodrug that greatly improves the oral bioavailability of 2-ME2 and thus provides a unique opportunity for more focused development of 2-ME2-PD1 as chemotherapy for BEAC.

Disclosure of Potential Conflict of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Kambhampati, R.A. Rajewski, I. Haque, A. Das, S. Banerjee
Writing, reviewing, and/or revising of the manuscript: S. Kambhampati, R.A. Rajewski, L. Yiannoutsos, R. Arajewski, I. Haque, S. Banerjee, D. Burns, E. B.–D. Reyes, P.J. Van Veldhuizen, S.K. Banerjee
Administrative, technical, or material support (i.e., reporting and organizing data, constructing databases): S. Kambhampati, R.A. Rajewski, M. Tanol, D. Burns, S.K. Banerjee

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Molecular Cancer Therapeutics

A Second-Generation 2-Methoxyestradiol Prodrug Is Effective against Barrett’s Adenocarcinoma in a Mouse Xenograft Model

Suman Kambhampati, Roger A. Rajewski, Mehmet Tanol, et al.


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