Synthesis and in Vivo Antitumor Evaluation of 2-Methoxyestradiol 3-Phosphate, 17-Phosphate, and 3,17-Diphosphate

Allison B. Edsall,[†] Gregory E. Agoston,[‡] Anthony M. Treston,[‡] Stacy M. Plum,[‡] Robert H. McClanahan,[§] Tian-Sheng Lu,[§] Wei Song,[§] and Mark Cushman*,[†]

Department of Medicinal Chemistry and Molecular Pharmacology, School of Pharmacy and Pharmaceutical Sciences, and the Purdue Cancer Center, Purdue University, West Lafayette, Indiana 47907, EntreMed, Inc., Rockville, Maryland 20850, and Department of Toxicology and Pharmacology, Ricera Biosciences, LLC, 7528 Auburn Road, Concord, Ohio 44077

Received June 4, 2007

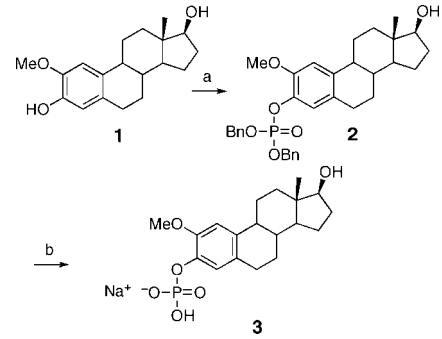
A prodrug strategy was investigated to address the problem of limited aqueous solubility and the resulting limited bioavailability of the antitumor agent 2-methoxyestradiol. The 3-phosphate, 17-phosphate, and 3,17-diphosphate of 2-methoxyestradiol were synthesized. 2-Methoxyestradiol 3-phosphate was metabolized more efficiently to the parent compound in vivo than 2-methoxyestradiol 17-phosphate, and it was also more cytotoxic in cancer cell cultures than either the 17-phosphate or the 3,17-diphosphate. These results agree with the in vivo anticancer activity of 2-methoxyestradiol 3-phosphate in a mouse Lewis lung carcinoma experimental metastasis model as opposed to the 17-phosphate and 3,17-diphosphate, both of which were inactive. The in vivo antitumor activity of 2-methoxyestradiol 3-phosphate at a dose of 200 mg/kg per day was comparable to that of a maximally tolerated dose of cyclophosphamide.

The endogenous human estrogen metabolite 2-methoxyestradiol (**1**), formed by hepatic cytochrome P450 2-hydroxylation of β-estradiol followed by 2-O-methylation by catechol *O*-methyltranseferase,[1–3] has attracted interest because of its potent inhibition of tumor vasculature and tumor cell growth.[4] Because solid tumor growth is dependent on angiogenesis, the potent antiangiogenic activity and tubulin polymerization inhibition of 2ME2 in vivo are of potential therapeutic value and have warranted further investigation in clinical trials.[4–10]

The cytotoxicity of 2-methoxyestradiol has been associated with uneven chromosome distribution, inhibition of mitosis, and an increase in the number of abnormal metaphases.[5,6] Competitive binding studies with [^{3}H]colchicine have demonstrated that the inhibitory effect of 2-methoxyestradiol on tubulin polymerization is mediated through the colchicine binding site on tubulin.[11,12] The inhibition of tubulin polymerization by 2-methoxyestradiol results in the inhibition of hypoxia inducible factor-1 (HIF) at the post-transcriptional level.[13] This dysregulation of HIF downstream from the 2ME2/tubulin interaction inhibits the transcriptional activation of HIF response genes, including vascular endothelial growth factor (VEGF), a major participant in the process of angiogenesis.[13]

There are many ways to improve the bioavailability of clinical agents. 2-Methoxyestradiol in its original capsule formulation displayed low plasma concentrations relative to the oral doses administered. This indicates low bioavailability of 2-methoxyestradiol, possibly as a function of its low aqueous solubility.[10,14] To address this issue from a formulation standpoint, EntreMed has developed an alternate formulation that is currently under clinical evaluation in oncology. An alternative approach is to prepare water-soluble prodrugs that would be efficiently metabolized to 2-methoxyestradiol as presented in this paper. Ample precedent exists for the successful use of phosphate prodrugs to increase aqueous solubility, including applications with estramustine, epinine, anandamide, buparvaquone, camptothecin, paclitaxel, etoposide, cortisone, resveratrol, and combretastatin A-4.[15–24] Phosphate prodrugs are typically converted to the parent molecule via nonspecific alkaline or acidic phosphatases.[15,17,20,22] This approach has allowed intravenous administration of phosphate prodrugs, including some steroid hormones, to overcome otherwise bleak pharmacokinetic profiles. In particular, combretastatin A-4, another microtubule-targeting and antiangiogenic agent, has recently benefited from a prodrug approach.[22,25]

Scheme 1[a]



[a] Reagents and conditions: (a) (1) DMAP, CH_3CN, CCl_4, DIEA, 0 °C (30 min); (2) dibenzylphosphite, 0 °C to room temperature (16 h); (b) (1) Pd/C, H_2, MeOH, 40 psi, room temperature (24 h); (2) 60 psi, room temperature (24 h).

Chemistry

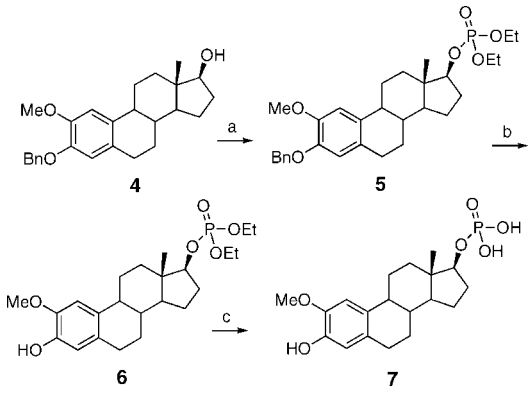
Three potential phosphate prodrugs of 2-methoxyestradiol were synthesized as described in Schemes 1–3. These were the 3-phosphate **3** (Scheme 1), the 17-phosphate **7** (Scheme 2), and the 3,17-diphosphate **9** (Scheme 3) derivatives.

The regioselective phosphorylation of 2-methoxyestradiol at the 3-hydroxyl group as opposed to the 17-hydroxyl group relied on the selective deprotonation of the phenol under basic conditions, resulting in the more nucleophilic phenoxide

* Author to whom correspondence should be addressed [telephone (765) 494-1465; fax (765) 494-6790; e-mail cushman@pharmacy.purdue.edu].
† Purdue University.
‡ EntreMed.
§ Ricera Biosciences, LLC.

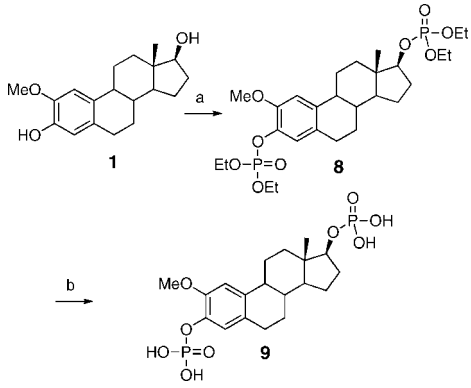
10.1021/jm070639e CCC: $37.00
Published on Web 12/06/2007

Scheme 2[a]



[a] Reagents and conditions: (a) Ti(*t*-BuO)$_4$, Et$_3$N, (EtO)$_2$P(O)Cl, CH_2Cl_2, room temperature (24 h); (b) Pd/C, H_2, MeOH, room temperature (17 h); (c) (1) TMSBr, $CHCl_3$, room temperature, (30 h); (2) MeOH, reflux (3 h).

Scheme 3[a]



[a] Reagents and conditions: Ti(*t*-BuO)$_4$, Et$_3$N, (EtO)$_2$P(O)Cl, CH_2Cl_2, room temperature (11 h); (b) (1) TMSBr, $CHCl_3$, room temperature (9 h); (2) MeOH.

anion. Treatment of 2-methoxyestradiol (**1**) with dimethylaminopyridine, carbon tetrachloride, diisopropylethylamine, and dibenzyl phosphite successfully produced the phosphoric acid dibenzyl ester **2** in high yield (Scheme 1). Hydrogenolysis of the two benzyl moieties of **2** provided the 3-phosphate analogue **3**.

To allow phosphorylation of the 17-hydroxyl group and not the 3-hydroxyl group, 2-methoxyestradiol (**1**) was first benzylated regioselectively on the 3-hydroxy group to afford 2-methoxyestradiol-3-benzyl ether (**4**),[26] which was then phosphorylated at the 17-position using titanium *tert*-butoxide, triethylamine, and diethylchlorophosphate to afford the diethylphosphate ester **5** (Scheme 2).[27] The titanium *tert*-butoxide was prepared by heating *tert*-butyl acetate and titanium isopropoxide at reflux.[28] Hydrogenolysis of the benzyl ether **5** yielded intermediate **6**. Reaction of the diethyl phosphate **6** with trimethylsilyl bromide afforded the desired 17-phosphate **7**.

The diphosphate intermediate **8** (Scheme 3) was synthesized utilizing methods similar to those employed for the preparation of intermediate **5**. Phosphorylation of the 3- and 17-hydroxyl groups of 2-methoxyestradiol (**1**) using titanium *tert*-butoxide, triethylamine, and diethylchlorophosphate provided intermediate **8**. Cleavage of the ethyl moieties with trimethylsilyl bromide in refluxing MeOH provided the desired 3,17-diphosphate **9**.

Biological Results and Discussion

The three phosphates **3**, **7**, and **9**, as well as their synthetic precursors **6** and **8**, were examined for antiproliferative activity

Table 1. Cytotoxicities of 2-Methoxyestradiol and Analogues (GI_{50} in Micromolar)[a]

no.	lung HOP-62	colon HCT-116	CNS SF-539	melanoma UACC-62	ovarian OVCAR-3	renal SN12C	prostate DU-145	breast MDA-MB-435	MGM[b]
1	0.70	0.47	0.32	0.36	0.21	0.95	1.80	0.08	1.3
3	0.69	1.45	0.66	1.32	0.34	1.31	2.95	0.11	2.7
6	ND[c]	19.0	19.5	14.4	15.1	17.4	ND	13.2	16.2
7	ND	87.1	75.9	38.0	ND	>100	ND	20.0	31.6
8	21.9	20.4	19.5	11.5	55.0	16.6	13.8	56.2	12.3
9	>100	>100	ND	>100	>100	>100	>100	>100	93.3

[a] The GI_{50} values are the concentrations affording *50%* growth inhibition of the individual human cancer cell lines. [b] MGM is the mean-graph midpoint and approximates the average concentration causing growth inhibition in all of the human cancer cell lines tested. [c] ND indicates that the value was not determined.

Table 2. Concentrations of 2-Methoxyestradiol 3-Phosphate (**3**), 2-Methoxyestradiol (**1**), and 2-Methoxystrone in Rat Plasma after Intravenous Administration of 1 mg/kg of 2-Methoxyestradiol 3-Phosphate

	concentration (ng/mL)		
time (min)	2ME2-3P[a]	2ME2[b]	2ME1[c]
0	BLQ[d]	BLQ	BLQ
5	366.4 ± 14.8[e]	153.4 ± 13.8	16.3 ± 2.7
15	31.3 ± 5.5	57.3 ± 13.0	12.7 ± 1.4
30	7.1 ± 2.0	22.3 ± 0.1	8.8 ± 1.5
60	1.2 ± 1.6	5.3 ± 4.0	2.7 ± 3.8
120	BLQ	BLQ	BLQ

[a] 2-Methoxyestradiol 3-phosphate. [b] 2-methoxyestradiol. [c] 2-methoxyestrone. [d] Below the limit of quantitation (1 ng/mL for 2ME2-3P and 5 ng/mL for 2ME2 and 2ME1). [e] Each value is the average of three separate determinations.

Table 3. Concentrations of 2-Methoxyestradiol 17-Phosphate (**7**), 2-Methoxyestradiol (**1**), and 2-Methoxystrone in Rat Plasma after Intravenous Administration of 1 mg/kg of 2-Methoxyestradiol 17-Phosphate

	concentration (ng/mL)		
time (min)	2ME2-17P[a]	2ME2[b]	2ME1[c]
0	BLQ[d]	BLQ	BLQ
5	1316.1 ± 73.1[e]	34.1 ± 5.0	BLQ
15	351.4 ± 120.0	15.0 ± 3.9	BLQ
30	137.7 ± 59.3	9.2 ± 2.0	BLQ
60	21.2 ± 12.8	BLQ	BLQ
120	6.9 ± 5.8	BLQ	BLQ

[a] 2-Methoxyestradiol 17-phosphate. [b] 2-methoxyestradiol. [c] 2-methoxystrone. [d] Below the limit of quantitation (1 ng/mL for 2ME2-17P and 5 ng/mL for 2ME2 and 2ME1). [e] Each value is the average of three separate determinations.

against the human cancer cell lines in the National Cancer Institute screen, in which the activity of each compound was evaluated with approximately 55 different cancer cell lines of diverse tumor origins.[29,30] The GI_{50} values obtained with selected cell lines, along with the mean graph midpoint (MGM) values, are summarized in Table 1. The MGM is based on a calculation of the average GI_{50} for all of the cell lines tested (approximately 55) in which GI_{50} values below and above the test range (10^{-8}–10^{-4} molar) are taken as the minimum (10^{-8} molar) and maximum (10^{-4} molar) drug concentrations used in the screening test. For comparison purposes, the activity of 2-methoxyestradiol (**1**) is also included in Table 1.

Comparison of the MGM values of the three phosphates reveals that the 3-phosphate **3** (MGM = 2.7 μM) is the most cytotoxic, followed by the 17-phosphate **7** (MGM = 31.6 μM) and the 3,17-diphosphate (MGM = 93.3 μM). The overall cytotoxicity of the 3-phosphate **3** is comparable to that of the parent compound 2-methoxyestradiol (**1**), although it was slightly less potent overall. Of the 52 cell lines that both compounds **1** and **3** were tested in successfully, 2-methoxyestradiol was more potent in 38 cell lines, its 3-phosphate was more potent in 12 cell lines, and in 2 cell lines the potencies were equal. Both the diethylphosphate synthetic precursor **6** and the bis(diethylphosphate) **8** were more potent than the corresponding phosphates **7** and **9**.

The pharmacokinetics of 2-methoxyestradiol (**1**), its 3-phosphate **3**, and its 17-phosphate **7** were investigated in rats. The 3-phosphate **3** and 17-phosphate **7** were not effective as prodrugs in improving the oral bioavailability of 2-methoxyestradiol in rats (data not shown). The pharmacokinetics were therefore studied after intravenous (iv) administration, and the results are listed in Tables 2–4. 2-Methoxyestradiol 3-phosphate (**3**), its metabolite 2-methoxyestradiol (**1**), and 2-methoxystrone (metabolite of 2ME2) were apparent at the earliest time point evaluated following administration of the 3-phosphate **3** (Table 2). The level of 2-methoxyestradiol (**1**) surpassed that of the prodrug **3** 15 min after administration and remained at higher concentration thereafter. The concentrations of all three compounds (**1**, **3**, and 2-methoxystrone) approached the lower limits of detection 30 min after administration, and they were undetectable after 1 h.

Table 4. Concentrations of 2-Methoxyestradiol and 2-Methoxystrone in Rat Plasma after Intravenous Administration of 1 mg/kg of 2-Methoxyestradiol

	concentration (ng/mL)	
time (min)	2ME2[a]	2ME1[b]
0	BLQ[c]	BLQ
5	763.5 ± 256.1	6.3 ± 8.9
15	168.0 ± 10.1	BLQ
30	44.7 ± 3.2	5.4 ± 0.2
60	11.3 ± 3.3	BLQ
120	BLQ	BLQ

[a] 2-Methoxyestradiol. [b] 2-methoxystrone. [c] Below the limit of quantitation (1 ng/mL for 2ME2-3P and 5 ng/mL for 2ME2 and 2ME1).

Table 5. Antitumor Activity of 2-Methoxy-β-estradiol 3-Phosphate in the LLC Experimental Metastasis Assay Following Intraperitoneal Administration

treatment group	dosing regimen	mean lung wt (g ±SD)	T/C[a]
vehicle control	0.1 mL qd × 12	0.60 ± 0.18	
2ME2	200 mg/kg qd[c] × 12	0.44 ± 0.17	0.55
2ME2-3P[b]	200 mg/kg qd × 12	0.23 ± 0.06	0.10
2ME2-3P	100 mg/kg qd × 12	0.44 + 0.21	0.55
cyclophosphamide	150 mg/kg qod[d] × 3	0.19 ± 0.03	0.02

[a] T/C was determined following correction for normal (non-tumor-bearing) lung weight as described in the Experimental Section. [b] 2-Methoxy-β-estradiol 3-phosphate. [c] qd, daily dosing. [d] qod, dosing every other day.

In contrast, the 17-phosphate **7** was metabolized to 2-methoxyestradiol much less efficiently (Table 3). The concentrations of 2-methoxyestradiol (**1**) achieved after iv administration of the 17-phosphate were significantly lower than those realized after administration of the 3-phosphate **3**, and the concentrations of 2-methoxyestradiol (**1**) never surpassed those of the prodrug **7**. The rate of disappearance of the 17-phosphate was slower than that of the 3-phosphate.

At the doses administered, the concentrations of 2-methoxyestradiol were higher after iv administration of 2-methoxyestradiol itself than after administration of either of the prodrugs (Table 4). The main advantage offered by the prodrugs is that they are more water soluble and can therefore be administered intravenously in higher doses.

The efficacies of 2ME2 and the two monophosphates **3** and **7**, as well as the diphosphate **9**, were examined in a mouse Lewis lung carcinoma (LLC) experimental metastasis model following intraperitoneal (ip) administration. In this experimental model, lung weight is used to indicate tumor burden. 2ME2 at a dose of 200 mg/kg resulted in lung weights corresponding to a decrease in tumor burden without morbidity being observed. 2-Methoxyestradiol 17-monophosphate (**7**) and 3,17-diphosphate (**9**) did not exhibit any effect on lung weight compared to the vehicle control-treated animals, indicating that neither compound demonstrates antitumor activity at the doses used (data not shown). In contrast, administration of 2-methoxyestradiol 3-phosphate (**3**) at doses of 100 and 200 mg/kg per day resulted in lung weights corresponding to a dose-related decrease in tumor burden (Table 5). At the higher dose of 200 mg/kg per day, the inhibition of tumor growth was comparable to that observed after administration of a maximally tolerated dosing regimen of cyclophosphamide (positive control treatment), and it is significantly better than that observed with 2-methoxyestradiol itself at the same dose. However, morbidity requiring

euthanasia was observed with both the 100 and 200 mg/kg doses of **3** (2/10 and 1/10, respectively) as opposed to 0% mortality with cyclophosphamide or with 2ME2. The fact that 2ME2-3P is more active than 2ME2 at the same dose suggests that 2ME2-3P has greater intrinsic activity than 2ME2 and/or that it is transported to the site of action more effectively than 2ME2 and is then hydrolyzed to 2ME2. The observed toxicity of 2ME2-3P will be addressed in any future development of this molecule.

The fact that 2-methoxyestradiol 3-phosphate (**3**) is active in vivo, whereas 2-methoxyestradiol 17-phosphate is inactive, agrees with the more efficient conversion of the 3-phosphate to the parent drug 2-methoxyestradiol (Tables 2 and 3). It also reflects the more potent activity of the 3-phosphate **3** versus the 17-phosphate **7** in the in vitro proliferation assays (Table 1).

Conclusion

The significance of the results of this study can be summarized in the following points: (1) Methods were developed for the efficient syntheses of 2-methylestradiol 3-phosphate, 2-methoxyestradiol 17-phosphate, and 2-methoxyestradiol 3,17-diphosphate. In principle, the routes to these three compounds are general and could be extended to the regioselective syntheses of the three possible phosphates of estradiol and a variety of its derivatives as well. (2) The three phosphates of 2-methoxyestradiol are not effective as prodrugs by the oral route of administration because they did not lead to increased levels of 2-methoxyestraiol when compared with administration of 2-methoxyestradiol. However, the prodrugs are more water soluble and can therefore be administered in higher doses than 2-methoxyestradiol by the intravenous route. This could increase the efficacy and versatility of 2-methoxyestradiol as an antitumor agent. (3) 2-Methoxyestradiol 3-phosphate (**3**) exhibits a significant, dose-related reduction in tumor weight in the Lewis lung carcinoma metastasis model following intraperitoneal administration to mice. The activity underscores the importance of continued development of 2-methoxyestradiol for treatment of cancer in humans. (4) 2-Methoxyestradiol 3-phosphate was metabolized more efficiently to the parent drug than the 17-phosphate after intravenous administration. This fact should be taken into consideration in the future design of phosphate prodrugs of estradiol and 2-methoxyestradiol analogues.

Experimental Section

2-Methoxy-β-estradiol-3-phosphoric Acid Dibenzyl Ester (2). 2-Methoxyestradiol (100 mg, 0.33 mmol) and dimethylaminopyridine (8 mg, 0.066 mmol) were dissolved in anhydrous acetonitrile (10 mL) in a flame-dried round-bottom flask under argon. The reaction mixture was cooled to 0 °C, and carbon tetrachloride (0.32 mL, 3.3 mmol) and diisopropylethylamine (0.29 mL, 1.65 mmol) were added via syringe. After 30 min, dibenzyl phosphite (0.22 mL, 1.00 mmol) was added, and the reaction mixture was allowed to warm to room temperature and stirred for 16 h. The reaction mixture was poured into a 0.5 M monobasic sodium phosphate solution (20 mL), and the aqueous mixture was extracted with ethyl acetate (3 × 50 mL). The combined organic layers were condensed in vacuo and purified via flash chromatography (silica gel, hexane/ethyl acetate 3:1–1:1 gradient) to provide **2** (165 mg, 89%) as a yellow oil: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.27 (m, 10 H), 6.80 (s, 1 H), 6.77 (s, 1 H), 5.12 (d, J = 7.79 Hz, 4 H), 3.70 (s, 3 H), 3.65 (t, J = 8.36 Hz, 1 H), 2.63 (m, 2 H), 2.22–1.09 (m, 14 H), 0.71 (s, 3 H); ^{31}P NMR (121.5 MHz, $CDCl_3$, 85% phosphoric acid standard) δ –5.26 (m, 1 P); IR (film) 3537, 2931, 1508, 1456, 1273, 1207, 1015, 934, 884, 738, 697 cm^{-1}; low-resolution ESIMS *m*/*z* (rel intensity) 563 (100, MH^+). Anal. $C_{33}H_{39}O_6P$: C, H.

2-Methoxy-β-estradiol 3-Phosphate Monosodium Salt (3). Palladium hydroxide, 20 wt % [10% palladium (dry basis) on carbon, wet, Degussa type, water ~50%, 0.011 g, 0.021 mmol] was added to a high-pressure vessel and covered with MeOH (5 mL). A solution of 2-methoxy-β-estradiol-3-dibenzylphosphate (2, 120 mg, 0.213 mmol) in MeOH (5 mL) was added to the vessel, and MeOH (5 mL) was used to rinse the residual palladium catalyst from the sides. The reaction mixture was shaken in a Parr hydrogenator under a 40 psi atmosphere of hydrogen for 24 h. Thin layer chromatography (TLC) indicated that no reaction had taken place. More palladium catalyst (20 mg, 0.038 mmol) was added, and the reaction mixture was shaken under a 60 psi atmosphere of hydrogen for 24 h. TLC indicated that the starting material had been completely converted to a product that could not be moved above the baseline on silica gel TLC in 5% MeOH/$CHCl_3$. The reaction mixture was filtered through a Celite pad, which was washed with MeOH. The organic solution was condensed in vacuo to provide a colorless oil. Water (20 mL) and diethyl ether (20 mL) were added, and the mixture was separated. The ether layer was washed with water (5 mL), and the combined water layers were frozen in a round-bottom flask. After freeze-drying, crude product was recovered as a fluffy white solid. A cellulose column was washed repeatedly (H_2O × 2, 0.1 M HCl × 2, H_2O × 2, 0.1 M NaOH × 2, H_2O × 2, MeOH × 2) to remove impurities from the column. (Note: a yellow impurity was removed during the base washing.) The crude product was purified via gravity chromatography (cellulose, MeOH), and the desired product was recovered from in vacuo condensation of the combined fractions to afford **3** (80 mg, 98%): mp (decomposed above 65 °C); ^{1}H NMR (300 MHz, DMSO) δ 7.01 (s, 1 H), 6.84 (s, 1 H), 3.70 (s, 3 H), 3.5 (t, J = 8.06 Hz, 1 H), 2.64 (m, 2 H), 2.27–1.01 (m, 16 H), 0.66 (s, 3 H); ^{31}P NMR (121.5 MHz, DMSO, 85% phosphoric acid standard) δ 4.70 (m, 1 P); IR (film) 3369, 2927, 2867, 2340, 1648, 1508, 1447, 1264, 1208, 1117, 962, 883 cm^{-1}; low-resolution ESIMS *m*/*z* (rel intensity) 383 (100, MH^+). Anal. $C_{19}H_{27}O_6Na \cdot 1.6H_2O$: C, H.

3-Benzyloxy-2-methoxy-β-estradiol-17-phosphoric Acid Diethyl Ester (5). Compound **4**[26] (500 mg, 1.27 mmol) was dissolved in anhydrous dichloromethane (10 mL) in a flame-dried round-bottom flask under an argon atmosphere. Titanium *tert*-butoxide[28] (0.05 mL, 0.13 mmol) was added, followed by the addition of triethylamine (0.27 mL, 1.91 mmol) and diethylchlorophosphate (0.18 mL, 1.27 mmol). The reaction mixture was allowed to stir at room temperature for 24 h before water (10 mL) was added. The aqueous mixture was extracted with ethyl acetate (3 × 25 mL), dried over anhydrous magnesium sulfate, and condensed in vacuo. Purification via flash chromatography (silica gel, hexane/ethyl acetate 3:1 by volume until the starting material eluted, followed by flushing the product from the column with acetone/hexane 1:1) gave the desired product **5** (468 mg, 89%) as a clear oil: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.46–7–29 (m, 5 H), 6.84 (s, 1 H), 6.62 (s, 1 H), 5.10 (s, 2 H), 4.30 (m, 1 H), 4.12 (m, 4 H), 3.86 (s, 3 H), 2.72 (m, 2 H), 2.26–1.22 (m, 13 H), 1.35 (m, overlapped, 6 H), 0.84 (s, 3 H); IR (film) 2929, 1607, 1515, 1455, 1262, 1025, 978, 740, 698 cm^{-1}; low-resolution ESIMS *m*/*z* (rel intensity) 529 (MH^+, 100). Anal. $C_{30}H_{41}O_6P$: C, H.

2-Methoxy-β-estradiol-17-phosphoric Acid Diethyl Ester (6). Palladium hydroxide, 20 wt % [20% palladium (dry basis) on carbon, wet, Degussa type, water ~50%, 0.015 g, 0.03 mmol] and **5** (150 mg, 0.28 mmol) were added to a round-bottom flask that had been evacuated and filled with argon three times. MeOH (5 mL) was added, and the reaction mixture was evacuated and filled with argon two times and then with hydrogen. After 17 h of stirring at room temperature, the reaction mixture was filtered through a Celite pad, which was washed with MeOH. The organic solution was condensed in vacuo to provide a clear oil. Purification via flash chromatography (silica gel, 1% MeOH in chloroform) gave the desired product **6** (118 mg, 96%) as a colorless solid: mp 106–108 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.78 (s, 1 H), 6.64 (s, 1 H), 5.46 (bs, 1 H), 4.30 (m, 1 H), 4.11 (m, 4 H), 3.86 (s, 3 H), 2.76 (m, 2 H), 2.28–1.16 (m, 13 H), 1.35 (m, overlapped, 6 H), 0.84 (s, 3 H); IR (film) 3271, 2928, 1509, 1262, 1028, 978 cm^{-1}; low-

resolution ESIMS *m/z* (rel intensity) 461 (MNa^+, 100). Anal. $C_{23}H_{35}O_6P \cdot 0.05CHCl_3$: C, H.

2-Methoxyestradiol 17-*O*-Phosphate (7). Trimethylsilyl bromide (1.20 mL, 9.13 mmol) was added via syringe to a solution of compound **6** (400 mg, 0.91 mmol) in anhydrous chloroform (5 mL). The reaction mixture was allowed to stir at room temperature for 30 h before it was condensed in vacuo. MeOH (5 mL) was added to the reaction flask, and the reaction mixture was heated at reflux for 3 h. After the reaction mixture had cooled, the solvent was evaporated in vacuo, and water (5 mL) was added to the solid residue. The turbid aqueous mixture was extracted with ethyl acetate (2 × 15 mL). The organic extracts were combined and evaporated in vacuo to give a pink solid. A cellulose column was washed repeatedly (H_2O × 2, 0.1 M HCl × 2, H_2O × 2, 0.1 M NaOH × 2, H_2O × 2, MeOH × 2) to remove impurities from the column. (Note: a yellow impurity was removed during the base washing.) The crude product was purified via gravity chromatography (cellulose, MeOH), and the desired product was recovered from in vacuo condensation of the combined fractions to afford **7** (320 mg, 92%): mp 82–85 °C; ^{1}H NMR [300 MHz, $(CD_3)_2CO$] δ 8.52 (bs, 3 H), 6.97 (s, 1 H), 6.65 (s, 1 H), 4.46 (m, 1 H), 3.86 (s, 3 H), 2.85 (m, 2 H), 2.91–1.32 (m, 13 H), 1.01 (s, 3 H); IR (KBr pellet) 3414, 2728, 1595, 1509, 1448, 1264, 1208, 1117, 1016, 873, 761, 511 cm^{-1}; low-resolution negative ESIMS *m/z* (rel intensity) 381 [$(M - H^+)^-$, 100]. Anal. $C_{19}H_{27}O_6P$: C, H.

2-Methoxy-β-estradiol-3,17-diphosphoric Acid Diethyl Ester (8). Compound **1** (5.0 g, 16.5 mmol) was dissolved in anhydrous dichloromethane (50 mL) in a flame-dried round-bottom flask under an argon atmosphere. Titanium *tert*-butoxide (1.28 mL, 3.31 mmol) was added, followed by triethylamine (8.05 mL, 57.75 mmol) and diethylchlorophosphate (5.96 mL, 41.25 mmol). The reaction mixture was allowed to stir at room temperature for 11 h before water (50 mL) was added. After the chlorinated organic layer was removed, the aqueous mixture was extracted with ethyl acetate (4 × 100 mL). The combined organic extracts were dried over anhydrous magnesium sulfate and condensed in vacuo. Purification via flash chromatography (silica gel, 500 g, ethyl acetate) yielded the desired product **8** (6.8 g, 72%) as a white solid: mp 65–68 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.96 (s, 1 H), 6.84 (s, 1 H), 4.31–3.82 (m, 9 H), 3.82 (d, J = 1.27 Hz, 3 H), 2.77–2.75 (m, 2 H), 2.28–1.18 (m, 25 H), 0.83 (s, 3 H); IR (film) 2981, 2931, 1509, 1273, 1033, 980, 920, 799 cm^{-1}; low-resolution ESI *m/z* (rel intensity) 575 (MH^+, 100). Anal. $C_{27}H_{44}O_9P_2$: C, H.

2-Methoxyestradiol 3,17-*O*,*O*-Diphosphate (9). Trimethylsilyl bromide (13.8 mL, 104.4 mmol) was added via syringe to a solution of compound **8** (4.0 g, 6.96 mmol) in anhydrous chloroform (50 mL). The reaction mixture was allowed to stir at room temperature for 9 h, and then it was condensed in vacuo. MeOH (50 mL) was added to the reaction flask, and the solvent was evaporated in vacuo using heat. Additional MeOH (50 mL) was added, and the solvent was evaporated again in vacuo using a heated water bath. The pink-brown mixture was taken up in ethyl acetate (20 mL) and extracted into deionized water (4 × 100 mL). Lyophilization of the water gave the pure product (2.8 g, 89%) as a pink solid: mp 165–169 °C; ^{1}H NMR [300 MHz, $(CD_3)_2CO$] δ 6.96 (s, 1 H), 6.95 (s, 1 H), 6.17 (bs, 7 H), 4.12 (dt, J = 8.8 and 8.07 Hz, 1 H), 3.83 (s, 3 H), 2.74 (m, 2 H), 1.87–0.91 (m, 13 H), 0.61 (s, 3 H); ^{31}P NMR [121.5 MHz, CD_3OD (85% phosphoric acid internal standard)] δ 0.87 (s, 1 H), 3.53 (m, 1 H); IR (KBr pellet) 3550, 2930, 1506, 1457, 1206, 1018, 873, 492 cm^{-1}; low-resolution ESIMS *m/z* (rel intensity) 463 (MH^+, 100). Anal. $C_{19}H_{28}O_9P_2 \cdot 1.52H_2O$: C, H.

Lewis Lung Carcinoma (LLC) Experimental Metastasis Model. Male, pathogen-free mice of the inbred mouse strain C57BL/6J were obtained from The Jackson Laboratory (Bar Harbor, ME). The mice were housed in a barrier environment under specific pathogen-free conditions and provided sterilized mouse chow (LM485, Harlan-Sprague–Dawley, Indianapolis, IN) and water ad libitum. LLC cells in exponential growth phase were harvested by a brief trypsinization (0.25% Difco trypsin and 0.02% EDTA for 1 min at 37 °C), washed, and resuspended to 5 × 10^5 cells/mL in Ca^{2+}, Mg^{2+} free PBS (BioWhittaker, Walkersville, MD). Six- to eight-week-old mice were injected with 0.2 mL of the cell suspension (1 × 10^5 tumor cells/mouse) via the lateral tail vein with a 27-gauge needle. Beginning 3 days after tumor cell injection, cohorts of mice (10 mice/treatment group) were treated intraperitoneally daily × 12 days with (a) 0.1 mL of vehicle control, (b) 100 or 200 mg/kg 2-methoxy-β-estradiol 3-phosphate monosodium salt, (c) 100 or 200 mg/kg 2-methoxyestradiol 17-*O*-phosphate, or (d) 100 or 200 mg/kg 2-methoxyestradiol 3,17-*O*,*O*-diphosphate. Following 12 days of treatment (15 days after tumor cell injection), mean lung weight was determined from each treatment group and corrected for non-tumor-bearing lung weight. Antitumor activity was then determined by calculating a treatment/control (*T*/*C*) value using the formula $T/C = (A - C)/(B - C)$, where A = mean lung weight of treated tumor-bearing mice, B = mean lung weight of vehicle control treated tumor-bearing mice, and C = mean lung weight of non-tumor-bearing mice.

Pharmacokinetics. Sprague–Dawley rats used in this study were obtained from Charles River Laboratories (Portage, MI) and were surgically implanted with jugular cannula to facilitate blood sampling. The animals were laboratory bred and were experimentally naive at the outset of the study.

The test articles, received as solids, were separately weighed into appropriate containers and formulated in 45% (w/v) hydroxypropyl-β-cyclodextrin (HPβCD) in water at concentrations of 0.5 mg/mL for intravenous injection, 1.25 mg/mL for oral administration, and 2.5 mg/mL for intraperitoneal administration. Formulations were prepared as close as possible to the time of dose administration.

Animals were observed once each morning and afternoon throughout the study for viability. Daily observations were recorded. Body weights were measured prior to dosing. The animals each received a single dose of the test compound. The dose volume for each compound was 2 mL/kg and was based on the most recent body weight obtained prior to dosing. Intravenous administration was via the tail vein. Animals were fasted overnight prior to dose administration. Predose samples were collected within 1 h of dosing. Following dosing, blood samples (ca. 200 μL) were collected into separate tubes containing anticoagulant (K-EDTA) via the jugular cannula at 2, 4, 6, 8, 12, and 24 h. For samples at 1 h and later, replacement saline was added via the jugular cannula. Blood samples were transferred to the study director or designate for preparation of plasma samples. Plasma samples were prepared as soon as possible after collection by centrifugation for 10 min using a tabletop centrifuge, immediately harvested, and stored at freezer temperatures (< −20 °C) pending analysis by LC-MS/MS. After the blood sampling, all animals were anesthetized with either carbon dioxide or carbon dioxide and oxygen, killed by opening the thoracic cavity, or killed by cervical dislocation and discarded.

For the preparation of standard curves, each analyte was spiked separately into 100 μL of rat plasma at various concentrations (5–2000 ng/mL for 2ME1, 5–2500 ng/mL for 2ME2, 1–1000 ng/mL for 2ME2-3P and 2ME2-17P), and vortex-mixed well. For standard curve samples and study samples, an aliquot of 10 μL of formic acid was added to each sample tube and mixed well. The samples were then extracted by protein precipitation using acetonitrile. The supernatant was then transferred and dried over a nitrogen flow under vacuum. The residue was reconstituted in 50:50 MeOH/H_2O and transferred to LC vial with low volume insert for LC-MS/MS analysis.

LC-MS/MS analysis was conducted using a PE Sciex API 3000 LC-MS/MS system. Electrospray ionization in negative ion MRM mode was conducted for quantitation of analytes. A Luna Phenyl-Hexyl HPLC column (150 × 2 mm, 5 μm particle size) was used. Mobile phase A was 5 mM NH_4OAc in H_2O, and mobile phase B was MeOH, and a linear step gradient (hold at 50% B for 0.3 min, ramp to 95% B in 3.2 min, hold at 95% B for 5.5 min, return to 50% B in 0.1 min) was used. The following precursor product ion pairs (MRM pairs) were used for quantitation: 2ME1 (299.4, 284.1), 2ME2 (301.3, 286.3), 2ME2 3-phosphate (381.2, 79.0), and 2ME2 17-phosphate (381.2, 79.0).

Supporting Information Available: Elemental analyses of compounds **2**, **3**, and **5**−**9**. This materials ia available free of charge via the Internet at http://pubs.acs.org.

References

(1) Breuer, H.; Knuppen, R. Biosynthesis of 2-methoxyestradiol in human liver. *Naturwissenschaften* **1960**, *12*, 280–281.

(2) Gelbke, H. P.; Knuppen, R. The excretion of five different 2-hydroxyestrogen monomethyl ethers in human pregnancy urine. *J. Steroid Biochem.* **1976**, *7*, 457–463.

(3) Fishman, J. A. Aromatic hydroxylation of estrogens. *Annu. Rev. Physiol.* **1983**, *45*, 61–72.

(4) Fotsis, T.; Zhang, Y.; Pepper, M. S.; Adlercreutz, H.; Montesano, R.; Nawroth, P. P.; Schweigerer, L. The endogenous oestrogen metabolite 2-methoxyoestradiol inhibits angiogenesis and suppresses tumour growth. *Nature* **1994**, *368*, 237–239.

(5) Seegers, J. C.; Aveling, M.-L.; van Aswegen, C. H.; Cross, M.; Koch, F.; Joubert, W. S. The cytotoxic effects of estradiol-17β, catecholestradiols and methoxyestradiols on dividing MCF-7 and HeLa cells. *J. Steroid Biochem.* **1989**, *32*, 797–809.

(6) Lottering, M.-L.; Haag, M.; Seegers, J. C. Effects of 17β-estradiol metabolites on cell cycle events in MCF-7 Cells. *Cancer Res.* **1992**, *52*, 5926–5932.

(7) Sledge, G. W.; Miller, K. D.; Haney, L. G.; Ngyyen, D. D.; Storniolo, A. M.; Phillips, E. N.; Pribluda, V.; Gubish, E. R. A Phase I Study of 2-Methoxyestradiol (2ME2) in Patients with Refractory Metastatic Breast Cancer (MBC), presented at the ASCO Annual Meeting, Orlando, FL, 2002.

(8) Miller, K. D.; Murry, D. J.; Curry, E.; Haney, L. G.; McClaskey, N.; Storniolo, A. M.; Phillips, E. N.; Pribluda, V.; Gubish, E. R.; Sledge, G. W. A Phase I Study of 2-Methoxyestradiol (2ME2) Plus Docetaxel in Patients with Metastatic Breast Cancer, presented at the ASCO Annual Meeting, Orlando, FL, 2002.

(9) Wilding, G.; Sweeney, C.; King, D. M.; Horvath, D.; Staab, M. J.; Fife, K.; Armstrong, V.; Sorioano, J. V.; Pribluda, V.; Gubish, E. R.; Sidor, C. Phase 2, Multicenter, Randomized, Double-Blind, Safety, Pharmacokinetic, Pharmacodynamic, and Efficacy Study of Two Doses of 2-Methoxyestradiol Administered Orally in Patients with Hormone Refractory Prostate Cancer, presented at the ASCO Annual Meeting, Orlando, FL, 2002.

(10) Dahut, W. L.; Lakhani, N. J.; Gulley, J. L.; Arlen, P. M.; Kohn, E. C.; Kotz, H.; McNally, D.; Parr, A.; Nguyen, D.; Yang, S. X.; Steinberg, S. M.; Venitz, J.; Sparreboom, A.; Figg, W. D. Phase I clinical trial of oral 2-methoxyestradiol, an antiangiogenic and apoptotic agent, in patients with solid tumors. *Cancer Biol. Ther.* **2006**, *5*, 22–27.

(11) D'Amato, R. J.; Lin, C. M.; Flynn, E.; Folkman, J.; Hamel, E. 2-Methoxyestradiol, an endogenous mammalian metabolite, inhibits tubulin polymerization by interacting at the colchicine site. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 3964–3968.

(12) Hamel, E.; Lin, C. M.; Flynn, E.; D'Amato, R. J. Interactions of 2-methoxyestradiol, an endogenous mammalian metabolite, with unpolymerized tubulin and with tubulin polymers. *Biochemistry* **1996**, *35*, 1304–1310.

(13) Mabjeesh, N. J.; Escuin, D.; LaValle, T. M.; Pribluda, V. S.; Swartz, G. M.; Johnson, M. S.; Willard, M. T.; Zhong, H.; Simons, J. W.; Giannakakou, P. 2ME2 inhibits tumor growth and angiogenesis by disrupting microtybules and dysregulating HIF. *Cancer Cell* **2003**, *3*, 363–375.

(14) Squillace, D. P.; Reid, J. M.; Kuffel, M. J.; Ames, M. M. Bioavailability and *in vivo* metabolism of 2-methoxyestradiol in mice. *Proc. Am. Assoc. Cancer Res.* **1998**, *39*, 523.

(15) Jordan, M. A. Mechanisms of action of antitumor drugs that interact with microtubules and tubulin. *Curr. Med. Chem.*−*Anti-Cancer Agents* **2002**, *2*, 1–17.

(16) Casagrande, C.; Merlo, L.; Santangelo, F. *N*-Methyldopamine-4-*O*-phosphate, an Orally Active Renal Vasodilator, presented at the 10th International Symposium in Medicinal Chemistry, Budapest, 1988; Abstract 87.

(17) Casagrande, C.; Merlo, L.; Ferrini, R.; Miragoli, G.; Semeraro, C. Cardiovascular and renal action of dopaminergic prodrugs. *J. Cardiovasc. Pharmacol.* **1989**, *14*, S40–S59.

(18) Mantyla, A.; Garnier, T.; Rautio, R.; Nevalainen, T.; Vepsalainen, J.; Koskinen, A.; Croft, S. L.; Jarvinen, T. Synthesis, in vitro evaluation, and antileishmanial activity of water-soluble prodrugs of buparvaquone. *J. Med. Chem.* **2004**, *47*, 188–195.

(19) Hanson, B. A.; Schowen, R. L.; Stella, V. J. A mechanistic and kinetic study of the E-ring hydrolysis and lactonization of a novel phosphoryloxymethyl prodrug of camptothecin. *Pharm. Res.* **2003**, *20*, 1031–1038.

(20) Juntunen, J.; Huuskonen, J.; Laine, K.; Niemi, R.; Taipale, H.; Nevalainen, T.; Pate, D. W.; Jarvinen, T. Anandamide prodrugs 1. Water-soluble phosphate esters of arachidonylethanolamide and R-methanandamide. *Eur. J. Pharm. Sci.* **2003**, *19*, 37–43.

(21) Freeman, S.; Ross, K. C. Prodrug design for phosphates and phosphonates. *Prog. Med. Chem.* **1997**, *34*, 111–147.

(22) Pettit, G. R.; Grealish, M. P.; Jung, M. K.; Hamel, E.; Pettit, R. K.; Chapuis, J. C.; Schmidt, J. M. Antineoplastic agents. 465. Structural modification of resveratrol: sodium resverastatin phosphate. *J. Med. Chem.* **2002**, *45*, 2534–2542.

(23) Vincent, L.; Kermani, P.; Young, L. M.; Cheng, J.; Zhang, F.; Shido, K.; Lam, G.; Bompais-Vincent, H.; Zhu, Z. P.; Hicklin, D. J.; Bohlen, P.; Chaplin, D. J.; May, C.; Rafii, S. Combretastatin A4 phosphate induces rapid regression of tumor neovessels and growth through interference with vascular endothelial-cadherin signaling. *J. Clin. Invest.* **2005**, *115*, 2992–3006.

(24) Pettit, G. R.; Temple, C.; Narayanan, V. L.; Varma, R.; Simpson, M. J.; Boyd, M. R.; Rener, G. A.; Bansal, N. Antineoplastic agents 322. Synthesis of combretastatin A-4 prodrugs. *Anti-Cancer Drug Des.* **1995**, *10*, 299–309.

(25) Kirwan, I. G.; Loadman, P. M.; Swaine, D. J.; Anthoney, D. A.; Pettit, G. R.; Lippert, J. W.; Shnyder, S. D.; Cooper, P. A.; Bibby, M. C. Comparative Preclinical pharmacokinetic and metabolic studies of the combretastatin prodrugs combretastatin A4 phosphate and A1 phosphate. *Clin. Cancer. Res.* **2004**, *10*, 1446–1453.

(26) Rao, P. N.; Cessac, J. W. A new, practical synthesis of 2-methoxylestradiols. *Steroids* **2002**, *67*, 1065–1070.

(27) Jones, S.; Selitsianos, D.; Thompson, K. J.; Toms, S. M. An improved method for Lewis acid catalyzed phosphoryl transfer with Ti(t-BuO)(4). *J. Org. Chem.* **2003**, *68*, 5211–5216.

(28) Campana, C. F.; Chen, Y.; Day, V. W.; Klemperer, W. G.; Sparks, R. A. Polyoxotitanates join the Keggin family: synthesis, structure and reactivity of $[Ti_{18}O_{28}H][OBu(t)]$ (17). *J. Chem. Soc., Dalton Trans.* **1996**, *691*, 702.

(29) Skehan, P.; Storeng, R.; Scudiero, D.; Monks, A.; McMahon, J.; Vistica, D.; Warren, J. T.; Bokesch, H.; Kenney, S.; Boyd, M. R. New colorimetric cytotoxicity assay for anticancer-drug screening. *J. Natl. Cancer Inst.* **1990**, *82*, 1107–1112.

(30) Boyd, M. R.; Paull, K. D. Some practical considerations and applications of the National Cancer Institute in vitro anticancer drug discovery screen. *Drug Dev. Res.* **1995**, *34*, 91–109.

JM070639E