

Phosphonoxyethyl Prodrug of Triptolide: Synthesis, Physicochemical Characterization, and Efficacy in Human Colon Adenocarcinoma and Ovarian Cancer Xenografts

Satish Patil,[†] Lev G. Lis,[†] Robert J. Schumacher,[‡] Beverly J. Norris,[‡] Monique L. Morgan,[‡] Rebecca A. D. Cuellar,[†] Bruce R. Blazar,[‡] Raj Suryanarayanan,[§] Vadim J. Gurvich,[†] and Gunda I. Georg^{*,†}

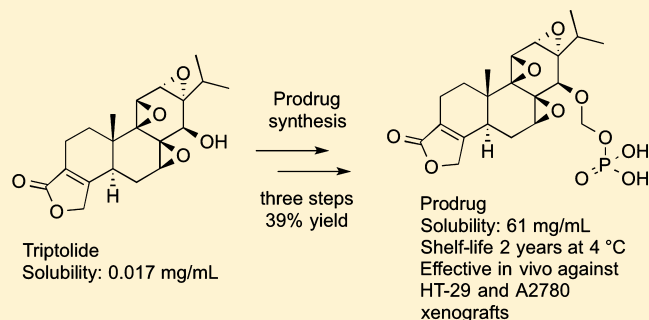
[†]Department of Medicinal Chemistry and Institute for Therapeutics Discovery and Development, College of Pharmacy, University of Minnesota, 717 Delaware Street SE, Minneapolis, Minnesota 55414, United States

[‡]Center for Translational Medicine, Academic Health Center, University of Minnesota, 420 Delaware Street SE, Minneapolis, Minnesota 55455, United States

[§]Department of Pharmaceutics, College of Pharmacy, University of Minnesota, 308 Harvard Street SE, Minneapolis, Minnesota 55455, United States

Supporting Information

ABSTRACT: A disodium phosphonoxyethyl prodrug of the antitumor agent triptolide was prepared from the natural product in three steps (39% yield) and displayed excellent aqueous solubility at pH 7.4 (61 mg/mL) compared to the natural product (17 μ g/mL). The estimated shelf life (t_{90}) for hydrolysis of the prodrug at 4 °C and pH 7.4 was found to be two years. In a mouse model of human colon adenocarcinoma (HT-29), the prodrug administered intraperitoneally was effective in reducing or eliminating xenograft tumors at dose levels as low as 0.3 mg/kg when given daily and at 0.9 mg/kg when given less frequently. When given via intraperitoneal and oral routes at daily doses of 0.6 and 0.9 mg/kg, the prodrug was also effective and well tolerated in a mouse model of human ovarian cancer (A2780).



INTRODUCTION

Triptolide (**1**, Figure 1), a diterpene triepoxide, was first isolated from the medicinal plant *Tripterygium wilfordii* Hook F (TWHF) and structurally characterized in 1972.¹ In the following 43 years, it has been reported to be active in vitro by inhibiting proliferation and inducing apoptosis of various cancer cell lines and as preventing tumor growth and metastases in vivo.² More recently, triptolide has been found to be effective against various cancers such as pancreatic cancer,³ neuroblastoma,⁴ breast cancer,⁵ and prostate cancer.⁶

The mechanisms of action of triptolide have been extensively investigated,^{7,8} and evidence has been provided that triptolide can covalently modify proteins presumably by epoxide ring opening reactions.^{1,2,9–11} It was recently shown that triptolide inhibits the ATPase activity of human XPB (xeroderma pigmentosum B) by covalently binding Cys342 of XPB to the 12,13-epoxide.^{9,10} Because XPB is part of transcription factor THFIIH, RNA II polymerase-mediated transcription and DNA excision repair are inhibited.⁹ Many of the observed anticancer effects can be explained by this mechanism, but other mechanisms¹² have been observed such as epigenetic modifications, suppressing kinases, and Hsp70 expression.^{3,13,14} Triptolide was also reported to be the first DCTPP1 (dCTP pyrophosphatase) inhibitor.¹⁵ It is of interest to note that this is

a noncovalent interaction of triptolide with the target protein. Furthermore, the compound has multiple biological activities that could have value in other therapeutic areas.^{2,16} Triptolide was reported to stimulate polycystin-2 channel opening, thereby restoring calcium signaling and resulting in attenuation of cyst formation in a mouse model of polycystic kidney disease.¹⁷ It is also known for its reversible male antifertility effects.¹⁸ In addition, triptolide was shown to preserve cognitive function in transgenic mouse models of Alzheimer's disease.^{19,20} The anticancer and other activities of triptolide such as its immunosuppressive and anti-inflammatory properties are more thoroughly described in recent reviews.^{6,7,12} Recent studies focus on the development of triptolide and its derivatives as potential antileukemic²¹ and antineoplastic agents.^{22,23}

Despite its promising bioactivities, poor aqueous solubility, dose-dependent toxicity, narrow therapeutic window, and lack of patent protection of triptolide are impediments to its preclinical development and clinical success. Two early stage clinical trials of triptolide as a potential drug for rheumatoid arthritis were conducted in the US over a decade ago.^{24,25} The

Received: August 27, 2015

Published: November 24, 2015

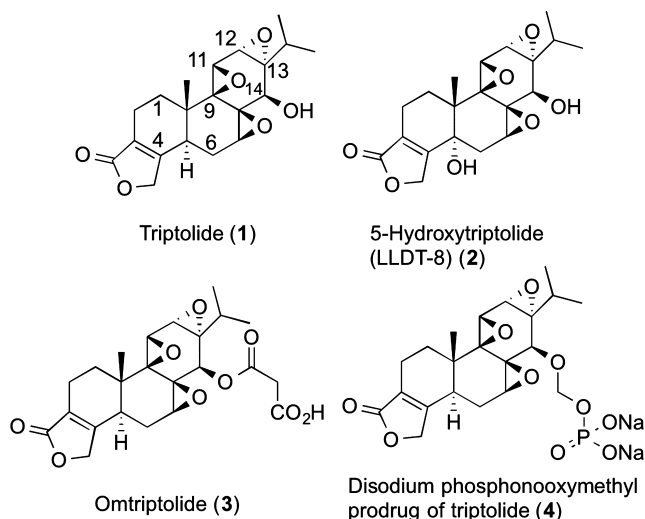


Figure 1. Structures of triptolide (1), 5-hydroxytriptolide (2), omtriptolide (3), and the disodium phosphonoxyethyl prodrug of triptolide (4).

5-hydroxytriptolide derivative 5-hydroxytriptolide (LLDT-8, 2, Figure 1) is currently in phase I clinical trial in China for rheumatoid arthritis.¹⁶

Prodrug strategies, involving carboxylic and amino acid esters, have been utilized previously with the intent to achieve desirable water solubility of triptolide.^{26,27} While several prodrugs of triptolide have been reported in the literature, triptolide succinate (omtriptolide, F6008, PG490-88, 3, Figure 1) is the only one reported to have entered clinical trials.²⁸ Unfortunately, the cleavage of the prodrug moiety was slow and incomplete, and significant interpatient variability was reported.²⁹ This does not discount the prodrug strategy but suggests that other prodrugs that provide a reliable release of triptolide are needed. A nontoxic, water-soluble, chemically stable, and patentable prodrug approach would be a viable option to overcome some of the physicochemical limitations of triptolide for the clinical development of this natural product.

Historically, the use of a phosphate group as a promoity has successfully overcome numerous delivery problems of potential drugs.^{30–32} These prodrugs are formed by either direct linkage of a phosphate moiety onto a hydroxyl group of a parent drug in the form of a phosphomonoester or by attaching it to the parent drug via a chemical linker. The phosphate promoity is ionized at physiological pH, resulting in a significant increase in aqueous solubility of poorly soluble phenol- and alcohol-bearing parent drugs.^{33–36} Additionally, these phosphomonoester prodrugs are typically stable with long shelf-lives and undergo an alkaline phosphatase (EC 3.1.3.1)-catalyzed bioconversion in vivo to release the parent alcohol or phenol drug and inorganic phosphate. Numerous phosphomonoester

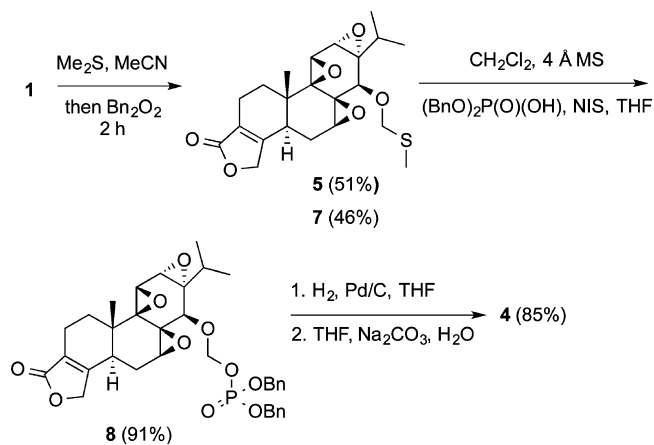
prodrugs have shown good in vitro^{37–39} and in vivo^{40–42} conversion to the parent drug in the presence of alkaline phosphatases.

The aforementioned omtriptolide was ineffective in clinical trials due to its incomplete and slow bioconversion in vivo.²⁹ We hypothesized that the succinate promoity directly attached to the 14-OH group of triptolide would not be easily accessible for enzymatic cleavage due to steric crowding. Therefore, the prerequisites for a novel prodrug strategy of triptolide were three-fold: enhanced aqueous solubility, chemical stability, and fast, complete bioconversion in vivo. We aimed to achieve these objectives by incorporating the phosphonoxyethyl promoity, as it possesses a favorable combination of high aqueous solubility and chemical stability. This strategy has been successful for solubility enhancement of paclitaxel and propofol, and, in both cases, the promoity was attached to a sterically hindered hydroxyl group.⁴³ Previously, the synthesis and evaluation of the phosphonoxyethyl derivative of triptolide 4 (Figure 1) for pancreatic cancer^{44,45} and several other preclinical cancer models has been reported.^{46–49} We are now describing an improved synthesis for 4, its physicochemical characterization, and its pharmacodynamic evaluation in human colon adenocarcinoma and ovarian cancer xenografts via intraperitoneal and oral routes and using less frequent dosing schedules than employed in previous studies.

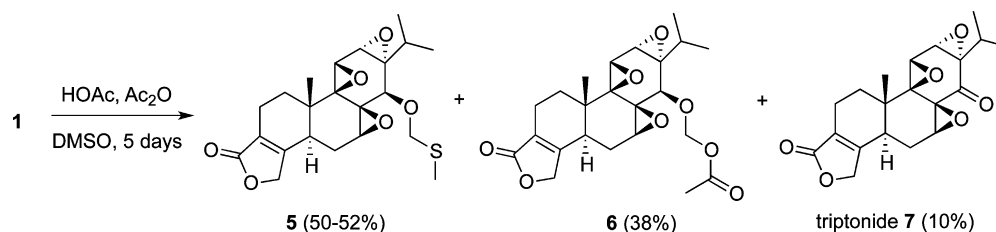
RESULTS AND DISCUSSION

Synthesis. Our initial attempts to prepare 4 in two steps from triptolide by either O-alkylation with chloromethyl phosphate diesters or direct alkylation of the hydroxyl group with chloriodomethane were not successful.^{35,50,51} Therefore, an alternative strategy⁴⁴ based on methylthiomethylation of the hydroxyl group was selected (Schemes 1 and 2) to furnish key

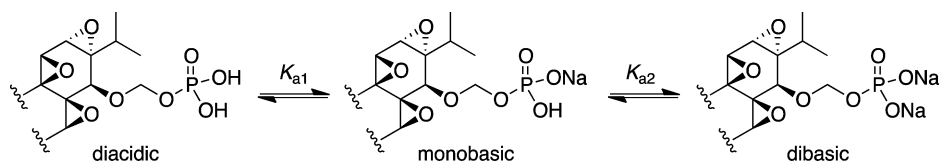
Scheme 2. Synthesis of 4



Scheme 1. Initial Synthesis of Methylthioether 5



Scheme 3. Speciation of 4: Diacidic, Monobasic, and Dibasic States



intermediate **5**.⁵² We observed complete conversion of triptolide during the Pummerer rearrangement, but a mixture of products was obtained. The products were separated by column chromatography and identified by NMR. While the target methylthiomethyl ether **5** was the main component (50–52% yield), acetoxymethyl ether **6** was identified as a major byproduct (38% yield), and triptonide (**7**) formed in 10% (Scheme 1).

This composition is similar to what was previously observed for this type of reaction using phenylsulfonyl derivatives.⁵³ Our attempts to carry out a basic hydrolysis of the acetoxymethyl ether **6** resulted in decomposition of the product, but triptolide could be recovered by acidic hydrolysis of **6** in 75–80% yield. An alternative approach (Scheme 2) based on treating triptolide with dimethyl sulfide and benzoyl peroxide in acetonitrile resulted in a similar yield of compound **5** (51%) with triptonide as the only side product in 46%. This methodology, however, allowed for significant reduction of the reaction time from 5 days to 2 h and also made purification of **5** easier.

In the next step (Scheme 2), the conversion of methylthiomethyl ether **5** to dibenzyl phosphate **8** was achieved using an *N*-iodosuccinimide-mediated (NIS) nucleophilic displacement with dibenzyl phosphate in $\text{CH}_2\text{Cl}_2/\text{THF}$ in the presence of 4 Å molecular sieves.⁴³ While the yield was high (91%), chromatographic purification on silica gel was difficult because of pronounced decomposition due to instability of the benzylic esters of the phosphate. The 50–100 mg scale flash chromatography experiments revealed approximately 20% degradation. However, in larger scale preparations where contact between the compound and silica gel is required for a longer period of time, 60–100% degradation was observed depending on the amount of substrate and the level of deactivation of the silica gel. Attempts to use alternate sorbents such as Florisil or alumina, as well as triethylamine deactivation of silica gel, resulted in similar levels of degradation. It was not possible to avoid purification prior to hydrogenation due to the various sulfur derivatives formed during the course of the synthesis. These compounds, even in minute quantities, can poison the Pd/C catalyst used in the benzyl ether cleavage. This led to increased catalytic loading and longer reaction time, which in turn resulted in a larger number and quantity of impurities that contaminated the final product. Additionally, during the formation of dibenzyl phosphate **8**, 7–10% of succinimide was produced from the reagent NIS, which coeluted during column chromatography. An alternative purification methodology was developed utilizing a sequence of extractions that are described in Experimental Section, which removed most of the impurities while avoiding product decomposition. This methodology has proven reliable, efficient, and scalable. It results in the removal of most sulfur derivatives and succinimide without the use of chromatographic purification.

In the penultimate step, dibenzyl phosphate **8** was subjected to hydrogenation in the presence of 10% Pd/C. The resultant

phosphate was converted to **4** by treatment with sodium carbonate. The final composition of the product included up to 6% of triptolide as a result of hydrolysis during the final chemical step. Because the product decomposes during silica gel column chromatography, it was isolated in 99% purity by preparative HPLC using a C18 column. The material is highly hygroscopic and was stored under nitrogen or argon gas.

Physicochemical Characterization. Physicochemical characterizations of **4** were conducted to assess its solubility and chemical stability.

Aqueous Solubility. Adjusting solution pH can be a simple and effective method to increase the water solubility of a weakly acidic or basic injectable drug. However, pH adjustment is not a viable approach to increase the solubility of molecules such as triptolide that lack ionizable groups. For such compounds, various strategies including the use of cosolvents, surfactants, and complexing agents (for example, parenterally safe cyclodextrins) have been used to overcome aqueous solubility limitations. However, some of these techniques can contribute significantly to toxicity. Ideally phosphonooxymethyl prodrugs will have good water solubility, particularly at physiological pH. Avoiding pH extremes and/or cosolvent addition could allow for rapid parenteral infusion without the risk of drug precipitation. Using HPLC, the aqueous solubility (buffered to pH 7.4 with Tris at room temperature) of triptolide was determined to be 17 $\mu\text{g}/\text{mL}$ while that of **4** was 61 mg/mL . Thus, by prodrug formation, the solubility was enhanced 3600 times. We also estimated the second dissociation constant ($\text{p}K_{a2}$) of **4**. In solution, depending on the pH, the prodrug would exist in its diacidic, monobasic, or dibasic states as represented in Scheme 3. The $\text{p}K_{a1}$ has limited pharmaceutical relevance, because the pH of most physiologically acceptable solutions would ensure ionization of the diacidic species. However, the $\text{p}K_{a2}$ is significant given its influence on aqueous solubility, chemical stability, and effectiveness as an enzyme substrate to ensure bioconversion. Therefore, within the pharmaceutically relevant pH range, **4** would exist in an equilibrium between its monobasic and dibasic species. Thus, titration of an equilibrium mixture of these two species provides an acid ionization constant, $\text{p}K_{a2}$, whose value corresponds to the inflection point. Estimation of this $\text{p}K_{a2}$ using ^{31}P NMR is advantageous, as the ^{31}P isotope has high natural abundance (100%) and its chemical shift is indicative of the change in ionization state of the phosphorus moiety.⁵⁴ The ^{31}P NMR spectrum of **4** was obtained as a function of pH. The experimental data shown for the change in observed phosphorus chemical shifts of **4** in solutions at varying pH (Figure 2) was used to determine $\text{p}K_{a2}$ (eq 4, experimental part). The pH dependency of this observed chemical shift measurement resulted in a sigmoidal curve, which is typical of an acid–base titration. The $\text{p}K_{a2}$ value of **4** was found to be 6.61, which compares favorably to estimates from the literature.^{55–57} At pH 7.4, **4** would exist predominantly as a dianion, thus ensuring high solubility.

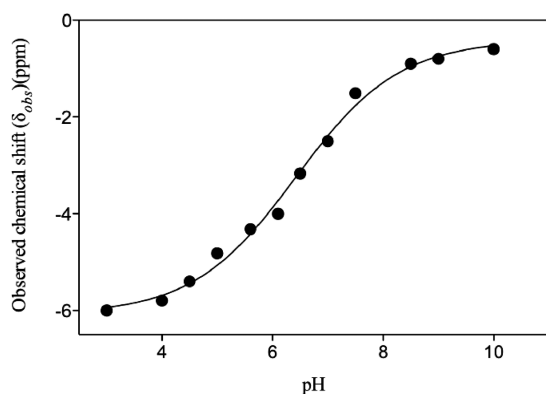


Figure 2. ^{31}P NMR titration curve for the second dissociation constant ($\text{pK}_{\text{a}2}$) of **4**. Curve fit of the experimental data to eq 4 (in Experimental Section) estimates the chemical shift of the monobasic (δ_{mb}) and dibasic (δ_{db}) fractions of **4** as -6.0 ppm and -0.62 ppm, respectively. The $\text{pK}_{\text{a}2}$ of **4** is 6.61 at 25°C .

Chemical Stability Determination. Following oral dosing, prodrugs are exposed to a wide pH range in the GI tract, from highly acidic in the stomach to neutral in the colon. Also, a wide array of hydrolytic enzymes, such as pepsin and pancreatin, are present in the GI tract. These enzymes have the natural function of digesting macromolecules for use as nutrients, but they can also bind and hydrolyze drug compounds. Therefore, prodrugs must be stable in the acidic, basic, and enzymatic conditions of the GI tract for in vivo oral dosing. The excipients in a formulation may also promote prodrug decomposition. Therefore, in vitro physiological stability of prodrugs in various buffers (pH 1–9) and in simulated gastrointestinal fluids should be assessed to gain insight into the stability of prodrugs. Thus, **4** was incubated with simulated gastric fluid (SGF), simulated intestinal fluid (SIF), and various buffers (pH 1–9). Oral dosing exposes compounds to pH 1–2 in the stomach, pH 4.5 at the beginning of the small intestine, pH 6.6 on average for the small intestine, and pH 5–7 in the colon. The gastric emptying time varies from 0.5–1 h in the fasted state to several hours after a heavy meal. SGF simulates stomach fluid and incorporates acidic and enzymatic hydrolysis conditions. SIF mimics the pH and hydrolytic enzymes in the intestine. Incubation of **4** in these solutions is a rapid way to determine if **4** would be stable under conditions found in the GI tract. Compound **4** demonstrated pH-dependent stability and rapidly degraded with conversion to triptolide in simulated gastric fluid and at very low pH values (1–2) and in simulated gastric fluid whereas it was markedly stable at higher pH values (3–9) and in simulated intestinal fluid (Table 1). The shorter half-life at low pH for **4** could

Table 1. In Vitro Stability of **4** in Various Buffers, SGF (Simulated Gastric Fluid), and SIF (Simulated Intestinal Fluid)

time (h)	percent of 4 remaining at indicated times							SGF	SIF
	pH 1	pH 2	pH 4	pH 6	pH 7.4	pH 9			
0	100	100	100	100	100	100	100	100	
1	89	98	100	100	100	100	93	100	
2	79	97	100	100	100	100	85	100	
4	62	95	100	100	100	100	71	100	
8	39	94	99	100	100	100	47	99	
24	0	83	99	99	100	100	0	99	

result in acid-mediated removal of the promoity in the stomach. However, **4** can be “protected” from the stomach acid by enteric coating.

Prodrugs, by their nature, tend to be less chemically stable than the parent drugs. To be practically useful, prodrugs must possess adequate chemical stability under the conditions of their use. Prodrugs intended for intravenous administration should exhibit adequate solution state stability so that a ready-to-use preparation with a reasonably long shelf life (i.e., 2 or more years) can be formulated. If the solution state stability is inadequate, the drug can be formulated as a dry powder (usually by lyophilization) and reconstituted into solution right before administration. For example, Safadi and co-workers described the development of phosphonoxyethyl carbonates as novel, water-soluble prodrugs of hindered alcohols. However, the lack of adequate chemical stability limited the commercial and clinical potential of this prodrug concept.³⁸ The chemical hydrolysis of **4** was followed at pH of 7.4 because the neutral pH range is perfectly suited for formulation delivery from a physiological perspective. We found that the hydrolysis of **4** followed pseudo-first-order kinetics. Using the pseudo-first-order rate constants, determined at several temperatures between 40 and 70°C (Table 2), the Arrhenius plot was drawn

Table 2. Pseudo-First-Order Rate Constants (k_{obsd}) of Hydrolysis of **4** at Different Temperatures

	40°C	50°C	60°C	70°C
rate constant (k_{obsd}) (h^{-1})	0.000494	0.001052	0.002482	0.008659

(not shown). From this plot, the activation energy (E_{a}) was calculated to be 63.4 kJ/mol. By extrapolating the plot to lower temperatures, the k_{obsd} at 25 and 4°C were obtained. This enabled the calculation of the half-life ($t_{1/2}$) and shelf life (t_{90}) of **4** at 25 and 4°C (Table 3).

Table 3. Extrapolated Pseudo-First-Order Rate Constant (k_{obsd}), for Hydrolysis of **4** at 25°C and at 4°C from Arrhenius Plot and the Calculated Half-life ($t_{1/2}$) and Shelf Life (t_{90})^a

	25°C	4°C
rate constant (h^{-1})	8.6×10^{-5}	6.5×10^{-6}
half-life ($t_{1/2}$) (years)	0.92	12.2
shelf life (t_{90}) (years)	0.14	1.9

^aShelf life (t_{90}) = time for 10% loss of **4**.

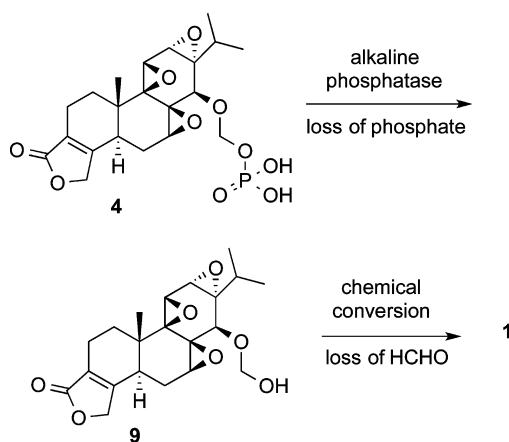
Thus, the chemical stability of **4** at neutral pH was found to be quite satisfactory giving an estimated shelf life of approximately 2 years (at 4°C). Therefore, the formulation of **4** in a freeze-dried form or as an aqueous solution at pH 7.4 is predicted to have an acceptable shelf life.

Enzymatic Conversion of **4 to Triptolide.** A prerequisite for a successful phosphate prodrug strategy is conversion catalyzed by alkaline phosphatase. In general, phosphomonoesters, such as phosphonoxyethyl prodrugs, are good substrates for both acid and alkaline phosphatases which are found in a wide variety of living organisms including bacteria, plants, and animals.⁵⁸ Alkaline phosphatase is found throughout the body and is mainly associated with plasma membranes of the intestine, placenta, bone, liver, and kidney at high concentrations.⁵⁹ This enzyme participates in hydrolase/

transferase reactions on a variety of phosphate-containing compounds under physiological conditions. For the *in vitro* evaluation of the dephosphorylation of **4**, alkaline phosphatase from bovine intestinal mucosa was chosen. As implied by its name, this enzyme has maximum activity in the alkaline region (pH 9.8). The *in vitro* assay for phosphate monoester prodrugs is typically performed at pH 9.8, an optimum pH for the activity of alkaline phosphatases. The evaluation of bioconversion kinetics is, however, more relevant if performed at physiological pH. Thus, a change in pH of reaction media to 7.4 may, in principle, reduce the catalytic efficiency of the enzyme. However, for proof-of-concept, confirming that **4** was indeed the substrate of alkaline phosphatase was paramount. Clearly, if **4** is administered parenterally in humans, the overwhelming presence of alkaline phosphate *in vivo* should guarantee bioconversion.

The *in vitro* enzymatic lability of **4** was recently reported by us.⁴⁴ We carried out the bioconversion of **4** into triptolide in the presence of alkaline phosphatase in glycine buffer at pH 9.8 (Scheme 4). Exposure of **4** to phosphatases cleaves the

Scheme 4. Conversion of 4 to Triptolide by Alkaline Phosphatase



phosphate group and releases the hydroxymethyl derivative **9**, which is chemically unstable and spontaneously releases triptolide and formaldehyde. The release of formaldehyde from prodrugs could raise a possible concern because of perceived toxicity.⁶⁰ However, it is well-known that the turnover of formaldehyde in the human body from endogenous formaldehyde production by normal metabolism and from exogenous exposure (for example from food) is in the range of 31–59 g per day.⁶⁰ Because prodrugs only release milligram amounts of formaldehyde from phosphonoxyethyl prodrugs per day, the small amount of formaldehyde adds very little to the baseline exposure of gram quantities produced by normal metabolism. In addition, given the short half-life of 1.5 min for formaldehyde, which is converted to formic acid, exposure to formaldehyde produced by a prodrug would be limited to approximately 10 min.⁶⁰

Both the disappearance of **4** and the formation of triptolide were measured. Compound **4** degradation was a first-order process. The half-life ($t_{1/2}$) of **4** was determined to be 2 min in the presence of alkaline phosphatase, showing rapid conversion of the modified drug into its parent compound. Additionally, **4** was found to be stable for 1 h in a similar assay conducted in the absence of alkaline phosphatase. The short half-life of **4** in

this assay indicates that the enzymatically catalyzed breakdown of **4** occurred at a fast rate. A short half-life for **4** is consistent with our hypothesis that a methylene-linked phosphate prodrug would be released rapidly and not be hampered by steric hindrance as seen with the succinate prodrug omtripotide. Steric hindrance has been noted in the bioconversion of a number of phosphate and phosphonoxyethyl prodrugs.^{37,61}

Evaluation of 4 for Efficacy in Mouse Models of Cancer. It was previously reported that compound **4** can reduce tumor growth, prevent tumor progression, and improve survival in multiple mouse models of pancreatic cancer.^{44,45} The efficacy of **4** has also been demonstrated in preclinical models of osteosarcoma, nonsmall cell lung carcinoma, human papillomavirus-positive head and neck squamous cell carcinoma, and ovarian cancer.^{46–49} These studies evaluated the efficacy of **4** after daily intraperitoneal (IP) administration. Here we used tumor xenograft models to evaluate the efficacy of **4** when administered on a less frequent schedule and when administered by the oral route.

Efficacy Study in a Mouse Model of Human Colon Adenocarcinoma. This study was designed to evaluate the potential efficacy and toxicity of **4** when administered IP daily (QD) over a range of doses and when administered on various schedules for 28 days. The animal model used in the study was the HT-29 human colon adenocarcinoma cell line implanted in female athymic nude mice as a subcutaneous tumor. Treatment groups with ten mice per group included four dose levels (0.1, 0.3, 0.6, and 0.9 mg/kg) and five dose regimens over the 4-week treatment period (daily [QD] for 4 weeks; QD for 2 weeks then no treatment for 2 weeks; QD for weeks 1 and 3 with no treatment during weeks 2 and 4; 3×/week for 4 weeks; QD for 2 weeks then 3×/week). Compound **4** administered daily IP was found to be effective in reducing or eliminating xenograft tumors of the human colon adenocarcinoma HT-29 in this animal model at dose levels from 0.3 to 0.9 mg/kg (Figure 3). Dose regimens in which 0.9 mg/kg of **4** was administered less frequently than daily or with a break from daily dosing were also found to be effective (Figure 4). In the groups that received 0.9 mg/kg daily for 4 weeks or 0.9 mg/kg daily for 2 weeks followed by 3×/week, all mice that survived until the end of the study (7/10 and 9/10, respectively) were tumor free. Compound **4** was generally well tolerated in female athymic nude mice with the only test-article related deaths observed in those mice receiving 0.9 mg/kg doses for at least 14 consecutive days (3 in the “QD for 4 weeks” group and 1 each in the “QD for 2 weeks” and the “QD for 2 weeks then 3×/week” groups). Clinical signs of toxicity were noted in some mice in higher dose groups after 14 days of dosing. The frequency of mortality and severity of adverse clinical signs escalated with dose level and frequency. The mice receiving a daily dose of 0.9 mg/kg had the least mean group weight gain and the highest incidence of skin irritation and necrosis toward the end of the study. Groups receiving a lower dose of **4** or 0.9 mg/kg on an intermittent schedule had fewer clinical signs of toxicity and higher weight gain over the course of the study.

Efficacy Study in a Mouse Model of Human Ovarian Cancer. This study was designed to evaluate the efficacy and tolerability of **4** in a mouse model of human ovarian cancer when administered via an intraperitoneal and oral route. The animal model used in this study was the A2780 human ovarian cancer cell line implanted in female athymic nude mice as a subcutaneous tumor. Treatments included daily doses of **4** ranging from 0.1 mg/kg to 1.2 mg/kg (ten mice per group).

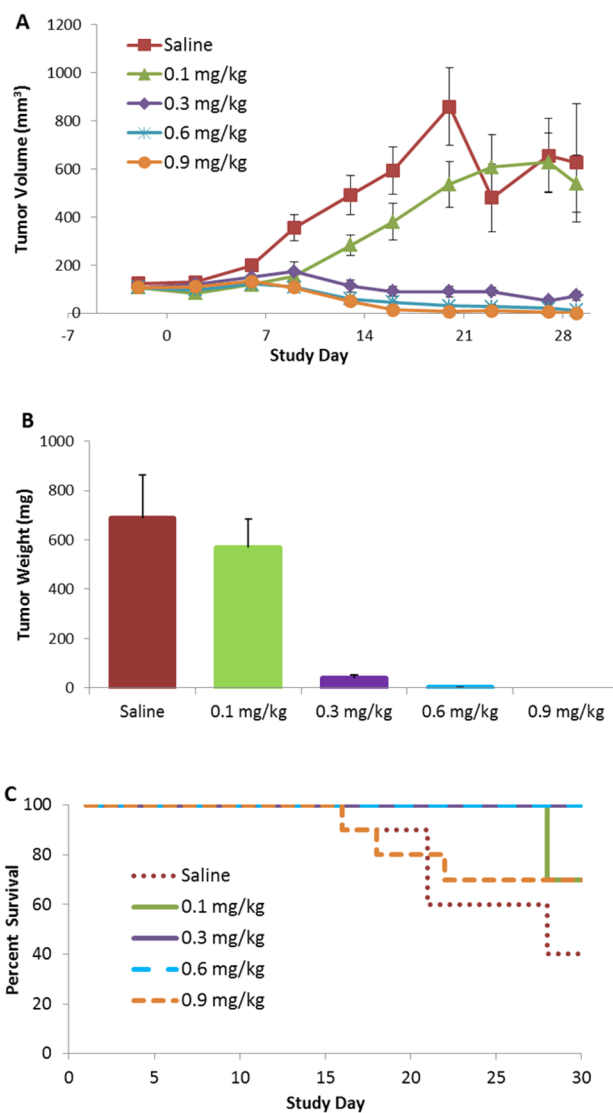


Figure 3. Efficacy and tolerability of **4** in a mouse model of human colon adenocarcinoma with daily IP administration. Compound **4** was delivered IP each day at the dose indicated. (A) Mean HT-29 solid tumor volume over time; (B) tumor weight at necropsy; (C) survival over time.

Administration of **4** by intraperitoneal injection was effective and well tolerated in female athymic nude mice with subcutaneous xenograft tumors of the human A2780 ovarian carcinoma at daily doses of 0.6 or 0.9 mg/kg (data not shown). Administration of **4** by oral gavage was also effective at daily doses of 0.6 or 0.9 mg/kg but with slightly higher rates of morbidity and mortality (Figure 5). Frequency of mortality and severity of adverse clinical signs escalated with dose level and only two mice in the 1.2 mg/kg oral dose group survived beyond day 5 (data not shown). Clinical signs of acute toxicity included anorexia, dehydration, and moribund condition or death. In the vehicle control group, 8 of 10 mice were euthanized before the end of the study because tumor volume had surpassed the tumor volume end point. However, in the 0.6 mg/kg and 0.9 mg/kg groups, most of the mice survived until the end of the study (7 of 10 and 6 of 10, respectively) and all but one in each group was tumor free.

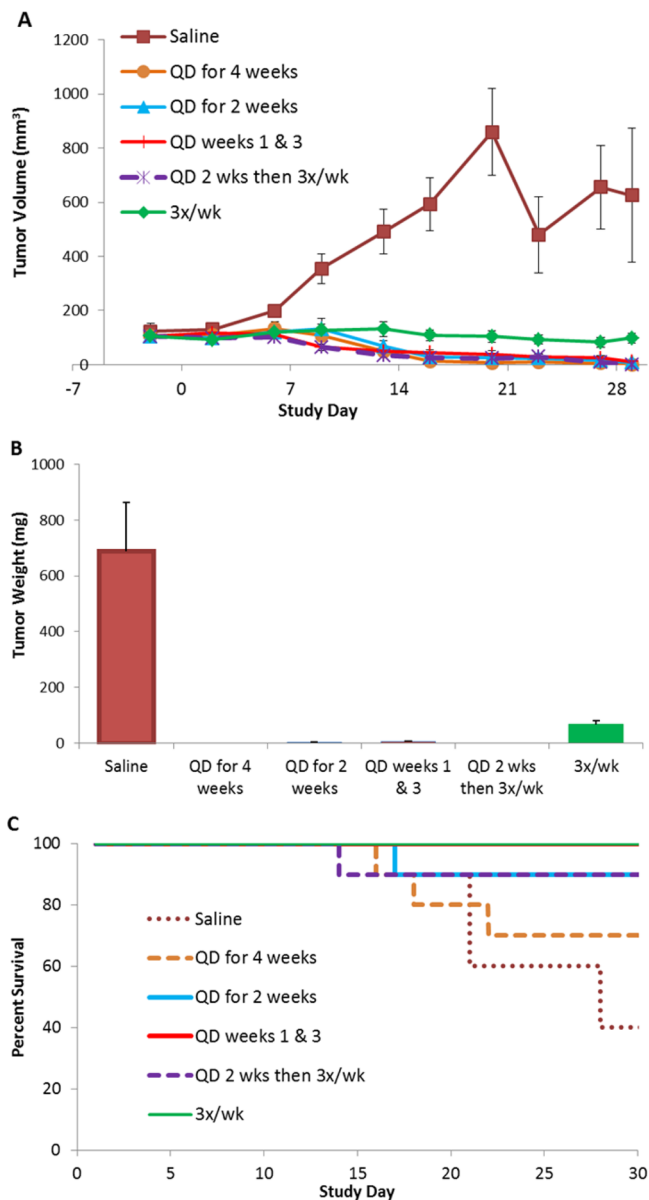


Figure 4. Efficacy and tolerability of **4** in a mouse model of human colon adenocarcinoma with intermittent dosing schedules. Compound **4** was administered IP at 0.9 mg/kg at the schedule indicated. (A) Mean HT-29 solid tumor volume over time; (B) tumor weight at necropsy; (C) survival over time.

CONCLUSIONS

To overcome solubility problems associated with the natural products, the disodium phosphonoxyethyl prodrug of triptolide was prepared. The synthesis, physicochemical characterization, and in vivo efficacy in mouse models of human colon adenocarcinoma and human ovarian carcinoma demonstrated that **4** has suitable properties to be a clinical candidate. Because the synthesis could be accomplished in three steps from the natural product, scale-up of this method for the clinical use of the prodrug does not pose a problem. The chemical stability of **4**, with a predicted shelf life of about 2 years at 4 °C, will allow storage of the prodrug over an extended time period. The in vivo mouse models of human colon adenocarcinoma and human ovarian carcinoma provide additional information about the efficacy and tolerability of **4**,

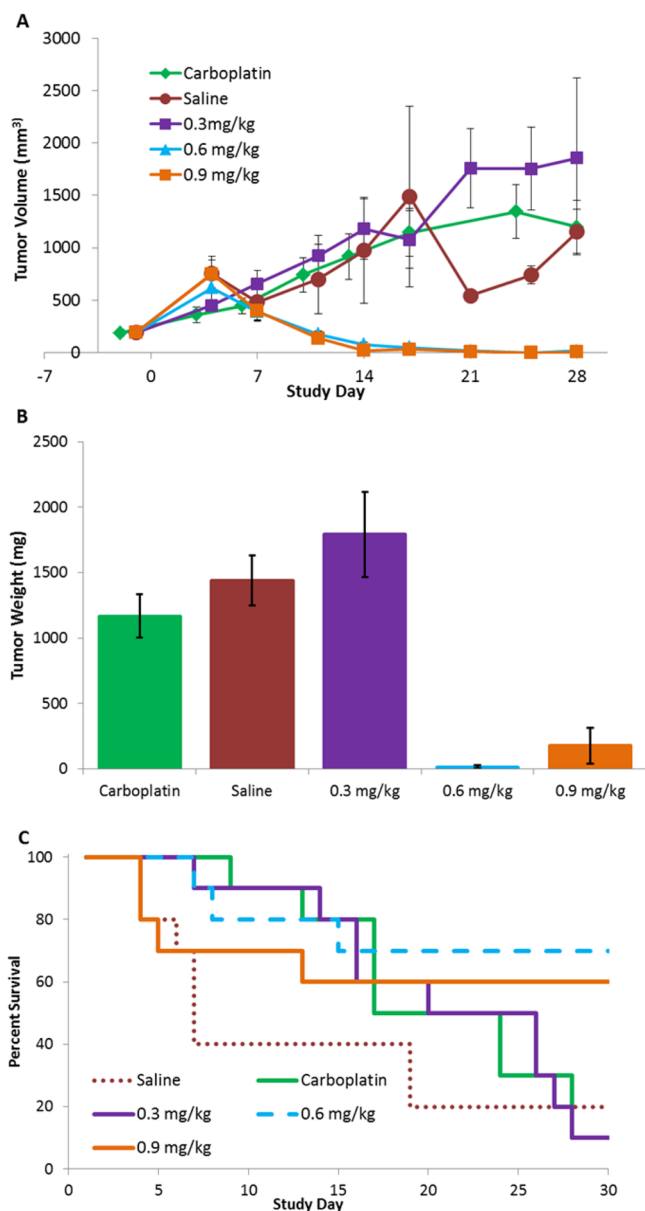


Figure 5. Efficacy and tolerability for compound 4 in a mouse model of human ovarian carcinoma with daily oral administration. Compound 4 was administered orally each day at the dose indicated. (A) Mean tumor volume over time; (B) tumor weight at necropsy; (C) survival over time.

suggesting that daily administration may not be required and that 4 may be effective when administered orally. After additional preclinical safety and toxicity testing, compound 4 entered phase I clinical trials in 2013 for evaluation in advanced gastrointestinal tumors.⁶²

EXPERIMENTAL SECTION

Chemistry. Unless otherwise specified, all materials, reagents, and solvents were obtained from commercial suppliers and were used without further purification. The progress of a synthetic procedure was monitored, where possible, by thin layer chromatography (TLC) and the compounds of interest were visualized by short-wave UV lamp or ceric ammonium molybdate stain. TLC was conducted on silica gel 250 μm , F254 plates. All solvents were removed using standard rotary evaporator techniques. Flash column chromatography was performed on silica gel. ¹H NMR spectra were recorded on a 400 MHz and ¹³C

NMR spectra were recorded on a 100 MHz spectrometer. Chemical shifts are reported in parts per million (ppm) using the solvent (CDCl_3) residual peak as the internal standard (for ¹H NMR: 7.27 ppm and for ¹³C NMR: 77.2 ppm). Coupling constants (*J*) are reported in hertz. The multiplicities of the signals are assigned using the following abbreviations: s = singlet, d = doublet, t = triplet, br = broad, m = multiplet. High-resolution mass spectrometry (HRMS) was performed by the University of Minnesota Mass Spectrometry Facility. The HPLC system used consisted of a Waters 2695 alliance HPLC with Waters 2996 photodiode Array detector (Milford, MA). The column used for analysis was a Phenomenex C18 (2) 150 \times 4.6 mm, 5 μm particle size column (Torrance, CA). For the analysis of triptolide and 4, the mobile phase consisted of acetonitrile (10% to 70% v/v) and a 10 mM sodium phosphate monobasic buffer solution adjusted to pH 7 with 1 N NaOH solution (90% to 30% v/v) for gradient elution over 20 min. This was pumped at a flow rate of 1.0 mL/min. The injection volume was 20 μL with detection at 218 nm. The retention times were 6.0 and 13.5 min for 4 and triptolide, respectively. The purity of 4 was >95%.

(5*b*S,6*a*S,7*a*R,8*R*,8*a*S,9*a*S,9*b*S,10*a*S,10*b*S)-8*a*-Isopropyl-10*b*-methyl-8-((methylthio)methoxy)-2,5,5*b*,6,6*a*,8,8*a*,9*a*,9*b*,10*b*-dodecahydrotris(oxireno)[2',3':4*b*,5;2'',3'':6,7;2''',3''':8*a*,9]phenanthro[1,2-*c*]furan-3(1*H*)-one (5). Procedure A. A solution of triptolide (1; 2.0 g, 5.6 mmol) in acetic acid (100 mL, 1.8 mol) and a solution of acetic anhydride (20 mL, 220 mmol) in DMSO (30 mL, 420 mmol) were mixed and stirred at room temperature for a period of 5 days. The reaction mixture was then poured into water (1 L) and neutralized with solid sodium bicarbonate added portionwise. The mixture was extracted with ethyl acetate (4 \times 250 mL), and the combined organic extract was dried over anhydrous sodium sulfate. The solvent was removed under reduced pressure. The oily residue was purified by flash chromatography (ethyl acetate–hexanes 1:5) to produce compound 5 (1.21 g; 52%) as a white foam. The NMR data matched those reported.²² HRMS calculated for ($\text{C}_{22}\text{H}_{28}\text{O}_6\text{SNa}$) required *m/z* [*M* + *Na*]⁺ 443.1505, found *m/z* 443.1507. In addition, acetoxymethyl ether 6 (896 mg, 38%) and triptonide 7⁶³ (191 mg, 10%) were isolated and identified.

((5*b*S,6*a*S,7*a*R,8*R*,8*a*S,9*a*S,9*b*S,10*a*S,10*b*S)-8*a*-Isopropyl-10*b*-methyl-3-oxo-1,2,3,5,5*b*,6,6*a*,8,8*a*,9*a*,9*b*,10*b*-dodecahydrotris(oxireno)[2',3':4*b*,5;2'',3'':6,7;2''',3''':8*a*,9]phenanthro[1,2-*c*]furan-8-yl)oxy)methyl Acetate (6). ¹H NMR (400 MHz, CD_2Cl_2) δ 0.80 (d, 3H, *J* = 6.9 Hz), 0.94 (d, 3H, *J* = 7.0 Hz), 1.05 (s, 3H), 1.21 (m, 1H), 1.53 (td, 1H, *J*₁ = 12.5, *J*₂ = 5.6, and *J*₃ = 1.3 Hz), 1.91 (t, 1H, *J* = 14.1 Hz), 2.06 (s, 3H), 2.14–2.27 (m, 4H), 2.68 (m, 1H), 3.30 (d, 1H, *J* = 5.4 Hz), 3.49 (d, 1H, *J* = 3.1 Hz), 3.58 (s, 1H), 3.77 (d, 1H, *J* = 3.1 Hz), 4.65 (m, 2H), 5.30 (d, 1H, *J* = 6.5 Hz), 5.44 (d, 1H, *J* = 6.5 Hz) ppm; ¹³C NMR (100 MHz, CD_2Cl_2) δ 14.0, 17.1, 17.4, 17.6, 21.4, 24.0, 27.2, 30.2, 36.3, 40.9, 55.3, 55.7, 60.5, 61.6, 64.1, 64.4, 70.6, 80.3, 89.7, 125.8, 160.7, 170.9, 173.7 ppm; HRMS calculated for ($\text{C}_{23}\text{H}_{29}\text{O}_8$) required *m/z* [*M* + *H*]⁺ 433.1862, found *m/z* 433.1850.

Procedure B. To a solution of triptolide (19.95 g, 55.36 mmol) in anhydrous acetonitrile (560 mL) at 0 $^\circ\text{C}$ dimethyl sulfide (31.8 mL, 433 mmol; 8 equiv) was added. Then benzoyl peroxide (53.7 g, 221 mmol, 4 equiv) was added portionwise during the course of 2 h. Thereafter, the reaction mixture was stirred at 0 $^\circ\text{C}$ for 2 h, diluted with ethyl acetate (1200 mL), washed with diluted sodium carbonate (saturated $\text{Na}_2\text{CO}_3 \cdot \text{H}_2\text{O}$, 1:2) (2 \times 150 mL) and brine (150 mL), and dried over sodium sulfate overnight. The solvent was then removed under reduced pressure, and the resulting viscous mass containing crystals was filtered through a glass filter and washed with cold ethyl acetate (50 mL). The collected solid was air-dried to yield triptonide (7; 7.01 g). The filtrate's volume was removed under reduced pressure, and the residue was dried overnight under high vacuum to produce 47.4 g of crude product. The crude product was purified by column chromatography on silica gel using EtOAc–hexanes (1:2) and then EtOAc– CH_2Cl_2 (1:1 followed by 2:1) mixtures as eluents to produce the target compound 5 (11.97 g; 51.4%) and additional amounts of triptonide (7; 2.10 g; the total yield of triptonide was 9.11 g, 46%).

Dibenzyl (((5*b*S,6*a*S,7*a*R,8*R*,8*a*S,9*a*S,9*b*S,10*a*S,10*b*S)-8*a*-Isopropyl-10*b*-methyl-3-oxo-1,2,3,5,5*b*,6,6*a*,8,8*a*,9*a*,9*b*,10*b*-dodecahydrotris(oxireno)[2',3':4*b*,5;2'',3'':6,7;2''',3''':8*a*,9]

phenanthro[1,2-c]furan-8-yl)oxy)methyl) Phosphate (8). To a solution of compound **5** (6.0 g; 14.3 mmol) in dry dichloromethane (240 mL) were added powdered 4 Å molecular sieves (6.0 g). The reaction mixture was placed under dry nitrogen, and then a solution of dibenzyl phosphate (4.78 g (17.2 mmol) and *N*-iodosuccinimide (3.86 g; 17.2 mmol) in anhydrous tetrahydrofuran (240 mL) was added slowly at 15–20 °C. After the addition was completed, the reaction mixture was stirred at rt for a period of 3 h, filtered, and diluted with dichloromethane (2400 mL). The solution was shaken with 0.1 M thiosulfate (240 mL) until fully decolorized and then washed with saturated sodium bicarbonate (240 mL) and brine (240 mL). The organic solution was dried over sodium sulfate for 0.5 h and filtered, and then the solvent was removed under reduced pressure. The residue was dissolved in anhydrous acetonitrile (900 mL), and the solution was extracted with pentane (4 × 200 mL). The acetonitrile solution was evaporated on a rotary evaporator followed by high vacuum overnight drying. Yield: 8.42 g (91%) of dibenzyl ester **8**, which was used directly in the next step without additional purification. The small sample was purified by silica gel flash chromatography (50% EtOAc/hexanes) to give compound **8** as white foam. ¹H NMR (400 MHz, CDCl₃) δ 0.72 (d, 3H, *J* = 6.8 Hz), 0.89 (d, 3H, *J* = 6.8 Hz), 1.05 (s, 3H), 1.27 (m, 1H), 1.48 (m, 1H), 1.82 (dd, 1H, *J*₁ = 14.7 and *J*₂ = 13.4 Hz), 2.03–2.35 (m, 4H), 2.64 (m, 1H), 3.14 (d, 1H, *J* = 5.5 Hz), 3.46 (d, 1H, *J* = 3.1 Hz), 3.65 (s, 1H), 3.76 (d, 1H, *J* = 3.1 Hz), 4.65 (m, 2H), 5.02 (m, 4H), 5.27 (m, 1H), 5.47 (m, 1H), 7.34 (m, 10H) ppm; ¹³C NMR (100 MHz, CDCl₃) δ 13.6, 16.8, 17.0, 23.3, 26.2, 29.62, 29.67, 35.7, 40.3, 54.7, 55.2, 59.3, 61.1, 63.6, 64.0, 69.36, 69.39, 69.42, 69.45, 69.9, 78.2, 92.9, 93.0, 125.5, 127.9, 128.0, 128.6, 135.5, 135.6, 160.1, 173.2 ppm; HRMS calculated for (C₃₅H₃₉O₁₀PNa) required *m/z* [M + Na]⁺ 673.2179, found *m/z* 673.2176. Analytical HPLC *t*_R = 8.5 min.

*Sodium (((5*b*S,6*a*S,7*a*R,8*R*,8*a*S,9*a*S,9*b*S,10*a*S,10*b*S)-8*a*-Isopropyl-10*b*-methyl-3-oxo-1,2,3,5,5*b*,6,6*a*,8,8*a*,9*a*,9*b*,10*b*-dodecahydrotris-(oxireno)[2',3':4*b*,5;2'',3'':6;7;2''',3''':8*a*,9]phenanthro[1,2-*c*]furan-8-yl)oxy)methyl Phosphate (Minnelide, **4**)*. To a 1 L round-bottom flask equipped with septa and stir bar were added Pd/C (1.8 g) and anhydrous tetrahydrofuran (10 mL). The flask was cooled in an ice bath and saturated with hydrogen gas using a bubbler under stirring up to full replacement of air. The bubbler was removed, and a solution of dibenzyl ester **8** (8.42 g; 12.94 mmol) in dry THF (700 mL) was added into the flask through a cannula while stirring and cooling the flask with an ice bath. Then the reaction mixture was saturated with hydrogen again and left under stirring in the hydrogen atmosphere for 3 h. The reaction mixture was monitored by TLC (EtOAc–hexanes, 3:2) until disappearance of dibenzyl ester **8**. After completion, the reaction mixture was purged with nitrogen and filtered through a pad of Celite. The THF solution was cooled to 10–12 °C and an ice cold solution of anhydrous sodium carbonate (1.235 g; 11.65 mmol) in deionized water (240 mL) was added slowly with stirring to keep the temperature below 18 °C. Then the solvents were removed on a rotary evaporator followed by high vacuum evaporation to give a slightly cloudy aqueous solution. The solution was placed into equipment for continuous extraction with dichloromethane and underwent extraction for 72 h. Then the aqueous solution was separated from CH₂Cl₂ and extracted with ethyl acetate (4 × 30 mL). Traces of organic solvent were removed by rotary evaporation, and the aqueous solution was passed through a 0.2 μm Acrodisc syringe filter to give a clear aqueous solution. The solution was freeze-dried, producing **4** (5.64 g; 85%). The product includes 6% of triptolide. Purification by preparative HPLC eluted with 15% methanol in water provides 99% pure **4** as a colorless hygroscopic powder. ¹H NMR (400 MHz, D₂O) δ 0.81 (d, 3H, *J* = 6.8 Hz), 1.00 (d, 3H, *J* = 6.8 Hz), 1.03 (s, 3H), 1.35 (m, 1H), 1.50 (m, 1H), 2.00 (dd, 1H, *J*₁ = 14.7 and *J*₂ = 13.4 Hz), 2.08–2.61 (m, 4H), 2.85 (m, 1H), 3.63 (d, 1H, *J* = 5.5 Hz), 3.81 (d, 1H, *J* = 3.1 Hz), 3.86 (s, 1H), 4.12 (d, 1H, *J* = 3.1 Hz), 4.92 (m, 2H), 5.07 (m, 2H) ppm; ¹³C NMR (100 MHz, D₂O) δ 12.9, 16.0, 16.3, 16.5, 22.3, 25.5, 28.9, 35.2, 39.8, 55.4, 56.1, 61.0, 61.5, 65.1, 65.5, 71.9, 77.6, 91.7, 123.8, 164.2, 177.3 ppm; HRMS calculated for (C₂₁H₂₆O₁₀P) required *m/z* [M + 1]⁺ 469.1264, found *m/z* 469.1267.

Aqueous Solubility Determination. Approximately 5 mg of each compound was weighed into 2 mL glass vials (in triplicate); 0.2 mL of Tris buffer (pH 7.4) was added for samples of **4** and 2 mL for triptolide samples. Each buffered solution was saturated with **4** or triptolide. The vials were then capped, sonicated, and vortexed prior to submersion in a constant temperature shaking bath (100 shakes/min maintained at 25 °C) for 24 h after which time excess solid drug was removed from the saturated solution by centrifugation and filtration. The clear filtrates were then sampled and appropriately diluted for quantification by HPLC.

Estimation of the Second Dissociation Constant (p*K*_{a2}) of **4 Using ³¹P NMR Chemical Shift.** A 4 mM solution of **4** was prepared in 10% D₂O in an isotonic solution to prepare stock solutions of 15 mL volume. Samples of varying pH were prepared by adding small volumes (μL) of 1 N HCl or 1 N NaOH solution and recording the resulting pH (Denver Instrument, Bohemia, NY). Aliquots (0.5 mL) were withdrawn from the stock solution after each alteration of pH and transferred to NMR tubes. Twelve to fifteen such samples for analysis were prepared in the pH range of 3–10. Solutions were analyzed by ³¹P NMR spectroscopy using a 400 MHz NMR instrument. The change in chemical shift was recorded as a function of pH. The ³¹P probe was calibrated using 85% H₃PO₄ as an external standard for the chemical shift.

In solution, depending on the pH and the dissociation constant (*K*_a), **4** exists in its diacidic, monobasic, or dibasic fractions as shown in Scheme 3. The fraction of **4** in its diacidic fraction may be ignored given the low and unlikely pH of such a solution within a physiological scenario. The fraction of **4** in the monobasic form (*f*_{mb}) and the fraction in the dibasic form (*f*_{db}) can, therefore, be expressed by eqs 1 and 2, respectively:

$$f_{mb} = \frac{[H^+]}{[H^+] + K_{a2}} \quad (1)$$

$$f_{db} = \frac{K_{a2}}{[H^+] + K_{a2}} \quad (2)$$

[H⁺] represents the molar hydrogen ion concentration. The observed chemical shift (δ_{obsd}) of the ³¹P signal is a product of the mole fraction of the prodrug species multiplied by its chemical shift and is expressed by eq 3.

$$\delta_{obsd} = f_{mb} \delta_{mb} + f_{db} \delta_{db} \quad (3)$$

δ_{mb} and δ_{db} represent the chemical shifts for the monobasic and the dibasic fraction of **4**, respectively. Substituting eqs 1 and 2 into 3, gives eq 4.

$$\delta_{obsd} = \frac{[H^+] \delta_{mb} + K_{a2} \delta_{db}}{[H^+] + K_{a2}} \quad (4)$$

The experimental data obtained indicate a shift in the ³¹P chemical shift with a variation in pH. This change in observed chemical shift as a function of pH is fit to eq 4 using the GraphPad Prism graphing software (Version 5.0, GraphPad Software, Inc., La Jolla, CA) to estimate the second dissociation constant (p*K*_{a2}) of **4**.

Stability Studies. A 20.0 mg amount of **4** was dissolved in Sodium Chloride Injection USP, and the volume was made up to 100 mL. Similarly, 3.00 mg of **4** was dissolved in Sodium Chloride Injection USP and the volume made up to 100 mL. These two constituted the “high” and “low” concentration formulations, respectively. Ampules were sterilized, to each ampule was added 0.5 mL of the solution of **4**, and then they were flame-sealed. Ampules were stored in ovens maintained at 70, 60, 50, and 40 °C. Solutions stored at 70 °C were sampled 4, 8, 10, 14, and 24 h after storage. Solutions stored at 60 °C were sampled 8, 14, 24, 32, 48, and 72 h after storage. Solutions stored at 50 °C were sampled 2, 4, 6, 8, and 11 days after storage. Solutions stored at 40 °C were sampled 4, 8, 13, 17, and 21 days after storage. The solutions were analyzed by HPLC as described in Experimental Section.

In Vitro Stability Study with 4. Simulated gastric fluid (SGF): The fluid was generated by preparing a solution of sodium chloride (0.20 g), concentrated HCl (0.70 mL), and pepsin (Sigma, P-7000, 0.32 g) in deionized water (95 mL). The final volume was adjusted to 100 mL by addition of deionized water. Simulated intestinal fluid (SIF): The fluid was generated by preparing a solution of monobasic potassium phosphate (KH_2PO_4 , 0.68 g), 0.1 N aqueous sodium hydroxide (38 mL), and pancreatin (Sigma, P-1625, 1.0 g) in deionized water (57 mL). The pH of the solution was adjusted to 7.5 by addition of 1 N aqueous sodium hydroxide, and then the final volume was adjusted to 100 mL by addition of deionized water.

Efficacy Studies in Mouse Xenograft Models. The source of cell lines for tumor xenografts was a cryopreserved vial supplied by ATCC. The HT-29 human colorectal adenocarcinoma cell line was established from the tumor tissue of a 44-year old adult female. Cells were grown as a monolayer in cell culture-treated disposable flasks and cultured in HyClone McCoy's 5A culture media supplemented with 10% v/v fetal bovine serum. The original vial was thawed and cultured to create a master cell bank (MCB). The MCB vials were tested for contamination by mycoplasma organisms and for a panel of selected rodent pathogens and confirmed negative prior to culture of cells for implantation in research animals. Mice were injected subcutaneously on the right flank with 1.74×10^6 HT-29 tumor cells in a solution of 50% Matrigel/50% unsupplemented McCoy's 5A culture medium in a volume of 0.1 mL using a 25 gauge needle.

The A2780 human ovarian cancer cell line was established from tumor tissue from an untreated patient. The source of the cell line for tumor xenografts in this study was a cryopreserved vial ordered from the ECACC (European Collection of Cell Cultures) via the vendor Sigma-Aldrich. Cells were grown as a monolayer in cell culture-treated disposable flasks and cultured in RPMI 1640 culture media supplemented with 10% v/v fetal bovine serum. The original vial was thawed and cultured to create an MCB. The MCB vials were tested for contamination by mycoplasma organisms and for a panel of selected rodent pathogens and confirmed negative prior to culture of cells for implantation in research animals. Mice were each injected subcutaneously on the right or left flank with 2.5×10^6 tumor cells in a solution of 50% Matrigel/50% unsupplemented RPMI 1640 culture medium in a volume of 0.1 mL.

Female athymic nude mice, age 7 to 9 weeks, were received from Taconic Farms (Albany, NY). The animals were examined and weighed on the day following receipt, and weights were measured and recorded twice weekly thereafter. All animals were allowed to acclimate to the laboratory environment for at least 7 days prior to subcutaneous injection of cancer cells. The average tumor volume at randomization was 110 mm^3 for the HT-29 study and 188 mm^3 for the A2780 study. There were 10 mice in each group, and the duration of treatment was 28 days for the HT-29 study and 30 days for the A2780 study. The dose concentration for each animal was based on the average of the body weight measurement for all of the animals in its respective dose group. The carboplatin dose in the A2780 study was 60 mg/kg administered twice each week. The test article was formulated in USP saline and delivered in a volume of 0.2 mL.

The following parameters and end points were evaluated: tumor volume, tumor volume changes, tumor weight at study end, clinical signs, survival, body weights, and body weight changes. Tumor measurements were performed at least twice weekly following the first appearance of tumors. A calibrated digital caliper was used to measure the length and width of each tumor. Tumor volume was calculated by the formula $L \times W^2/2$, where W is the smallest dimension recorded. Tumor volumes and tumor weights are expressed as mean \pm SEM.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acs.jmedchem.5b01329](https://doi.org/10.1021/acs.jmedchem.5b01329).

^1H NMR and ^{13}C NMR spectra for new compounds **6**, **8**, and **4** and ^{31}P NMR spectrum for compound **4** (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*Phone: 612-626-6320. E-mail: georg@umn.edu.

Author Contributions

All authors participated in drafting and/or revising the manuscript. Individual contributions: Satish Patil developed the synthesis of **4** and designed and carried out the stability studies and was involved in the analysis and interpretation of data. Lev Lis developed the scale-up synthesis method for **4**. Robert Schumacher designed and directed the in vivo studies and led the analysis and interpretation of in vivo study data. Beverly Norris conducted the in vivo studies and participated in the analysis and interpretation of in vivo study data. Monique Morgan conducted the in vivo studies and participated in the analysis and interpretation of in vivo study data. Rebecca Cuellar contributed to the analysis and interpretation of data. Bruce Blazar contributed to the development of the in vivo studies and to the analysis and interpretation of the in vivo study data. Raj Suryanarayanan participated in the stability studies and was involved in the analysis and interpretation of data. Vadim J. Gurvich was involved in development of the scale-up synthesis method for **4** and in the analysis and interpretation of data. Gunda Georg was involved in the design of the prodrug, its synthesis, the stability studies, and the analysis and interpretation of data.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors are grateful to the Masonic Cancer Center (NIH P30 CA77598), the Center for Translational Medicine, and the College of Pharmacy for generous funding provided for this research. These studies were also supported by the Vince and McKnight Presidential Chairs (to G.I.G.) and the William and Mildred Peters Endowment Fund (Raj S.). We thank Mary A. Smart for the purification of **4** by HPLC, Michael Maher and Vincent Lanctot for assistance with the xenograft studies, and Jing Wang for his help with the stability studies.

■ ABBREVIATIONS USED

C_{max} , maximal blood concentration; DCTPP1, (dCTP pyrophosphatase 1); E_a , activation energy; k_e , elimination rate constant; k_{obsd} , pseudo-first-order rate constant; k_r , reversion rate constant; MCB, master cell bank; NIS, *N*-iodosuccinimide; pK_{a2} , second dissociation constant; SD, standard deviation; SGF, simulated gastric fluid; SIF, simulated intestinal fluid; $t_{1/2}$, half-life; t_{90} , shelf life; USP, United States Pharmacopeia; XPB, xeroderma pigmentosum B; TFHII, transcription factor II H

■ REFERENCES

- (1) Kupchan, S. M.; Court, W. A.; Dailey, R. G.; Gilmore, C. J.; Bryan, R. F. Tumor inhibitors. LXXIV. Triptolide and triptodiolide, novel antileukemic diterpenoid triepoxides from *Tripterygium wilfordii*. *J. Am. Chem. Soc.* **1972**, *94*, 7194–7195.
- (2) Brinker, A. M.; Ma, J.; Lipsky, P. E.; Raskin, I. Medicinal chemistry and pharmacology of genus *Tripterygium* (Celastraceae). *Phytochemistry* **2007**, *68*, 732–766.
- (3) Phillips, P.; Dudeja, V.; McCarrroll, J.; Borja-Cacho, D.; Dawra, R.; Grizzle, W.; Vickers, S.; Saluja, A. Triptolide induces pancreatic cancer cell death via inhibition of heat shock protein 70. *Cancer Res.* **2007**, *67*, 9407–9416.
- (4) Antonoff, M. B.; Chugh, R.; Borja-Cacho, D.; Dudeja, V.; Clawson, K. A.; Skube, S. J.; Sorenson, B. S.; Saltzman, D. A.; Vickers,

- S. M.; Saluja, A. K. Triptolide therapy for neuroblastoma decreases cell viability in vitro and inhibits tumor growth in vivo. *Surgery* **2009**, *146*, 282–290.
- (5) Tan, B. J.; Tan, B. H.; Chiu, G. N. C. Effect of triptolide on focal adhesion kinase and survival in MCF-7 breast cancer cells. *Oncol. Rep.* **2011**, *26*, 1315–1321.
- (6) Liu, Q. Y. Triptolide and its expanding multiple pharmacological functions. *Int. Immunopharmacol.* **2011**, *11*, 377–383.
- (7) Sangwan, V.; Saluja, A. K. Mechanism of action of the anti-cancer agent triptolide. In *Neutraceuticals and Cancer*; Sarkar, F. H., Ed.; Springer: New York, 2012; pp 135–150.
- (8) Vispe, S.; DeVries, L.; Creancier, L.; Besse, J.; Breand, S.; Hobson, D. J.; Svejstrup, J. Q.; Annereau, J.-P.; Cussac, D.; Dumontet, C.; Guilbaud, N.; Barret, J.-M.; Bailly, C. Triptolide is an inhibitor of RNA polymerase I and II-dependent transcription leading predominantly to down-regulation of short-lived mRNA. *Mol. Cancer Ther.* **2009**, *8*, 2780–2790.
- (9) Titov, D. V.; Gilman, B.; He, Q.-L.; Bhat, S.; Low, W.-K.; Dang, Y.; Smeaton, M.; Demain, A. L.; Miller, P. S.; Kugel, J. F.; Goodrich, J. A.; Liu, J. O. XPB, a subunit of TFIIH, is a target of the natural product triptolide. *Nat. Chem. Biol.* **2011**, *7*, 182–188.
- (10) He, Q.-L.; Titov, D. V.; Li, J.; Tan, M.; Ye, Z.; Zhao, Y.; Romo, D.; Liu, J. O. Covalent modification of a cysteine residue in the XPB subunit of the general transcription factor TFIIH through single epoxide cleavage of the transcription inhibitor triptolide. *Angew. Chem., Int. Ed.* **2015**, *54*, 1859–1863.
- (11) McCallum, C.; Kwon, S.; Leavitt, P.; Shen, D.-M.; Liu, W.; Gurnett, A. Triptolide binds covalently to a 90 kDa nuclear protein. Role of epoxides in binding and activity. *Immunobiology* **2007**, *212*, 549–556.
- (12) Liu, Z.; Ma, L.; Zhou, G. B. The main anticancer bullets of the Chinese medicinal herb, Thunder God Vine. *Molecules* **2011**, *16*, 5283–5297.
- (13) MacKenzie, T. N.; Mujumdar, N.; Banerjee, S.; Sangwan, V.; Sarver, A.; Vickers, S.; Subramanian, S.; Saluja, A. K. Triptolide induces the expression of miR-142–3p: A negative regulator of Heat Shock Protein 70 and pancreatic cancer cell proliferation. *Mol. Cancer Ther.* **2013**, *12*, 1266–1275.
- (14) Antonoff, M. B.; Chugh, R.; Skube, S. J.; Dudeja, V.; Borja-Cacho, D.; Clawson, K. A.; Vickers, S. M.; Saluja, A. K. Role of Hsp-70 in triptolide-mediated cell death of neuroblastoma. *J. Surg. Res.* **2010**, *163*, 72–78.
- (15) Corson, T. W.; Cavga, H.; Aberle, N.; Crews, C. M. Triptolide directly inhibits dCTP pyrophosphatase. *ChemBioChem* **2011**, *12*, 1767–1773.
- (16) Zhou, Z.-L.; Yang, Y.-X.; Ding, J.; Li, Y.-C.; Miao, Z.-H. Triptolide: Structural modifications, structure-activity relationships, bioactivities, clinical development and mechanisms. *Nat. Prod. Rep.* **2012**, *29*, 457–475.
- (17) Leuenroth, S. J.; Okuhara, D.; Shotwell, J. D.; Markowitz, G. S.; Yu, Z.; Somlo, S.; Crews, C. M. Triptolide is a traditional Chinese medicine-derived inhibitor of polycystic kidney disease. *Proc. Natl. Acad. Sci. U. S. A.* **2007**, *104*, 4389–4394.
- (18) Lue, Y.; Sinha, H. A. P.; Wang, C.; Leung, A.; Baravarian, S.; Reutrakul, V.; Sangsawan, R.; Chaichana, S.; Swerdloff, R. S. Triptolide: A potential male contraceptive. *J. Androl.* **1998**, *19*, 479–486.
- (19) Cheng, S.; LeBlanc, K. J.; Li, L. Triptolide preserves cognitive function and reduces neuropathology in a mouse model of Alzheimer's disease. *PLoS One* **2014**, *9* (10), e108845 (Oct 2).
- (20) Wang, Q.; Xiao, B.; Cui, S.; Song, H.; Qian, Y.; Dong, L.; An, H.; Cui, Y.; Zhang, W.; He, Y.; Zhang, J.; Yang, J.; Zhang, F.; Hu, G.; Gong, X.; Yan, Z.; Zheng, Y.; Wang, X. Triptolide treatment reduces Alzheimer's disease (AD)-like pathology through inhibition of BACE1 in a transgenic mouse model of AD. *Dis. Models Mech.* **2014**, *7*, 1385–1395.
- (21) Fidler, J. M.; An, J.; Carter, B. Z.; Andreeff, M. Preclinical antileukemic activity, toxicology, toxicokinetics and formulation development of triptolide derivative MRx102. *Cancer Chemother. Pharmacol.* **2014**, *73*, 961–974.
- (22) Xu, H.; Tang, H.; Feng, H.; Li, Y. Design, synthesis and structure–activity relationships studies on the D ring of the natural product triptolide. *ChemMedChem* **2014**, *9*, 290–295.
- (23) Zhou, B.; Li, X. M.; Tang, H. Y.; Miao, Z. H.; Feng, H. J.; Li, Y. C. Total synthesis of novel D-ring-modified triptolide analogues: structure-cytotoxic activity relationship studies on the D-ring of triptolide. *Org. Biomol. Chem.* **2011**, *9*, 3176–3179.
- (24) Tao, X. L.; Cush, J. J.; Garret, M.; Lipsky, P. E. A phase I study of ethyl acetate extract of the Chinese antirheumatic herb *Tripterygium wilfordii* Hook F in rheumatoid arthritis. *J. Rheumatol.* **2001**, *28*, 2160–2167.
- (25) Tao, X. L.; Younger, J.; Fan, F. Z.; Wang, B.; Lipsky, P. E. Benefit of an extract of *Tripterygium wilfordii* Hook F in patients with rheumatoid arthritis - A double-blind, placebo-controlled study. *Arthritis Rheum.* **2002**, *46*, 1735–1743.
- (26) Dai, D.; Yuan, H.; Musser, J. H. Preparation of triptolide prodrugs having high aqueous solubility. WO 02070472, 2002.
- (27) Musser, J. H. Synthesis of triptolide prodrugs having high aqueous solubility for immunosuppressive and anti-inflammatory treatment. WO 0012483, 2000.
- (28) Fidler, J. M.; Li, K.; Chung, C.; Wei, K.; Ross, J. A.; Gao, M.; Rosen, G. D. PG490–88, a derivative of triptolide, causes tumor regression and sensitizes tumors to chemotherapy. *Mol. Cancer Ther.* **2003**, *2*, 855–862.
- (29) Kitzen, J. J. E. M.; de Jonge, M. J. A.; Lamers, C. H. J.; Eskens, F. A. L. M.; van der Biessen, D.; van Doorn, L.; ter Steeg, J.; Brandely, M.; Puozzo, C.; Verweij, J. Phase I dose-escalation study of F60008, a novel apoptosis inducer, in patients with advanced solid tumors. *Eur. J. Cancer* **2009**, *45*, 1764–1772.
- (30) Sinkula, A. A.; Yalkowsky, S. H. Rationale for design of biologically reversible drug derivatives: Prodrugs. *J. Pharm. Sci.* **1975**, *64*, 181–210.
- (31) Ellis, J. M.; Ross, J. W.; Coleman, C. I. Fosamprenavir - A novel protease inhibitor and prodrug of amprenavir. *Formulary* **2004**, *39*, 151–154.
- (32) Stella, V. J. A case for prodrugs: Fosphenytoin. *Adv. Drug Delivery Rev.* **1996**, *19*, 311–330.
- (33) Cho, M. J.; Kurtz, R. R.; Lewis, C.; Machkovech, S. M.; Houser, D. J. Metronidazole phosphate - a water-soluble prodrug for parenteral solutions of metronidazole. *J. Pharm. Sci.* **1982**, *71*, 410–414.
- (34) Loo, J. C.; Mcgilveray, I. J.; Jordan, N.; Brien, R. Pharmacokinetic evaluation of betamethasone and its water-soluble phosphate ester in humans. *Biopharm. Drug Dispos.* **1981**, *2*, 265–272.
- (35) Mantyla, A.; Garnier, T.; Rautio, R.; Nevalainen, T.; Vepsäläinen, 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.
- (36) Yoshimura, H.; Watanabe, K.; Oguri, K.; Fujiwara, M.; Ueki, S. Synthesis and pharmacological activity of a phosphate ester of delta-8-tetrahydrocannabinol. *J. Med. Chem.* **1978**, *21*, 1079–1081.
- (37) Kearney, A. S.; Stella, V. J. The in vitro enzymatic liabilities of chemically distinct phosphomonoester prodrugs. *Pharm. Res.* **1992**, *9*, 497–503.
- (38) Safadi, M.; Oliyai, R.; Stella, V. J. Phosphoryloxymethyl carbamates and carbonates - Novel water-soluble prodrugs for amines and hindered alcohols. *Pharm. Res.* **1993**, *10*, 1350–1355.
- (39) Tenhoor, C. N.; Stewart, B. H. Reconversion of fosphenytoin in the presence of intestinal alkaline-phosphatase. *Pharm. Res.* **1995**, *12*, 1806–1809.
- (40) Gunnarsson, P. O.; Andersson, S. B.; Johansson, S. A.; Nilsson, T.; Plymforshell, G. Pharmacokinetics of estramustine phosphate (Estracyt) in prostatic-cancer patients. *Eur. J. Clin. Pharmacol.* **1984**, *26*, 113–119.
- (41) Hare, L. E.; Yeh, K. C.; Ditzler, C. A.; McMahon, F. G.; Duggan, D. E. Bioavailability of dexamethasone 2. Dexamethasone phosphate. *Clin. Pharmacol. Ther.* **1975**, *18*, 330–337.

- (42) Mollmann, H.; Rohdewald, P.; Schmidt, E. W.; Salomon, V.; Derendorf, H. Pharmacokinetics of triamcinolone acetonide and its phosphate ester. *Eur. J. Clin. Pharmacol.* **1985**, *29*, 85–89.
- (43) Golik, J.; Wong, H. S. L.; Chen, S. H.; Doyle, T. W.; Wright, J. J. K.; Knipe, J.; Rose, W. C.; Casazza, A. M.; Vyas, D. M. Synthesis and antitumor evaluation of paclitaxel phosphonoxyethyl ethers: A novel class of water soluble paclitaxel pro-drugs. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 1837–1842.
- (44) Chugh, R.; Sangwan, V.; Patil, S. P.; Dudeja, V.; Dawra, R. K.; Banerjee, S.; Schumacher, R. J.; Blazar, B. R.; Georg, G. I.; Vickers, S. M.; Saluja, A. K. A preclinical evaluation of minnelide as a therapeutic agent against pancreatic cancer. *Sci. Transl. Med.* **2012**, *4*, 156ra139.
- (45) Banerjee, S.; Nomura, A.; Sangwan, V.; Chugh, R.; Dudeja, V.; Vickers, S.; Saluja, A. K. CD133+ tumor initiating cells (TIC) in a syngenic murine model of pancreatic cancer respond to minnelide. *Clin. Cancer Res.* **2014**, *20*, 2388–2399.
- (46) Rousalova, I.; Banerjee, S.; Sangwan, V.; Evenson, K.; McCauley, J. A.; Kratzke, R.; Vickers, S. M.; Saluja, A.; D'Cunha, J. Minnelide: A novel therapeutic that promotes apoptosis in non-small cell lung carcinoma In vivo. *PLoS One* **2013**, *8*, e77411.
- (47) Banerjee, S.; Thayanithy, V.; Sangwan, V.; Mackenzie, T. N.; Saluja, A. K.; Subramanian, S. Minnelide reduces tumor burden in preclinical models of osteosarcoma. *Cancer Lett.* **2013**, *335*, 412–420.
- (48) Caicedo-Granados, E.; Lin, R.; Fujisawa, C.; Yueh, B.; Sangwan, V.; Saluja, A. Wild-type p53 reactivation by small-molecule Minnelide™ in human papillomavirus (HPV)-positive head and neck squamous cell carcinoma. *Oral Oncol.* **2014**, *50*, 1149–1156.
- (49) Rivard, C.; Geller, M.; Schnettler, E.; Saluja, M.; Vogel, R. I.; Saluja, A.; Ramakrishnan, S. I. Inhibition of epithelial ovarian cancer by Minnelide, a water-soluble pro-drug. *Gynecol. Oncol.* **2014**, *135*, 318–324.
- (50) Ueda, Y.; Matiskella, J. D.; Golik, J.; Connolly, T. P.; Hudyma, T. W.; Venkatesh, S.; Dali, M.; Kang, S. H.; Barbour, N.; Tejwani, R.; Varia, S.; Knipe, J.; Zheng, M.; Mathew, M.; Mosure, K.; Clark, J.; Lamb, L.; Medin, I.; Gao, Q.; Huang, S.; Chen, C. P.; Bronson, J. J. Phosphonoxyethyl prodrugs of the broad spectrum antifungal azole, ravuconazole: Synthesis and biological properties. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 3669–3672.
- (51) Stella, V. J.; Zygmunt, J. J.; Georg, I. G.; Safadi, M. S. Water-soluble prodrugs of hindered alcohols or phenols. US 6451776, 2002.
- (52) Fiedler, J. M.; Leu, K. S. Method for treatment of severe acute respiratory syndrome. WO 020887 A2, 2005.
- (53) Trost, B. M.; Lynch, J.; Renaut, P.; Steinman, D. H. Enantiocontrolled cycloaddition approach to (+)-brefeldin-A. *J. Am. Chem. Soc.* **1986**, *108*, 284–291.
- (54) Albert, A.; Serjeant, E. P. *The determination of ionization constants. A laboratory manual*; Chapman and Hall: London, 1984.
- (55) Juntunen, J.; Huuskonen, J.; Laine, K.; Niemi, R.; Taipale, H.; Nevalainen, T.; Pate, D. W.; Jarvinen, T. Anandamide prodrugs. I. Water-soluble phosphate esters of arachidonylethanolamide and R-methanandamide. *Eur. J. Pharm. Sci.* **2003**, *19*, 37–43.
- (56) Kearney, A. S.; Stella, V. J. Hydrolysis of pharmaceutically relevant phosphate monoester monoanions: Correlation to an established structure–reactivity relationship. *J. Pharm. Sci.* **1993**, *82*, 69–72.
- (57) Kumler, W. D.; Eiler, J. J. The acid strength of mono and diesters of phosphoric acid. The n-alkyl esters from Me to Bu, the esters of biological importance and the natural guanidinephosphoric acids. *J. Am. Chem. Soc.* **1943**, *65*, 2355–2361.
- (58) Yalkowsky, S. H.; Krzyzaniak, J. F.; Ward, G. H. Formulation-related problems associated with intravenous drug delivery. *J. Pharm. Sci.* **1998**, *87*, 787–796.
- (59) Garattini, E.; Margolis, J.; Heimer, E.; Felix, A.; Udenfriend, S. Human placental alkaline phosphatase in liver and intestine. *Proc. Natl. Acad. Sci. U. S. A.* **1985**, *82*, 6080–6084.
- (60) Dhreshwar, S. S.; Stella, V. J. Your prodrug releases formaldehyde: Should you be concerned? No! *J. Pharm. Sci.* **2008**, *97*, 4184–4193.
- (61) DeGoey, D. A.; Grampovnik, D. J.; Flosi, W. J.; Marsh, K. C.; Wang, X. C.; Klein, L. L.; McDaniel, K. F.; Liu, Y. Y.; Long, M. A.; Kati, W. M.; Molla, A.; Kempf, D. J. Water-soluble prodrugs of the human immunodeficiency virus protease inhibitors lopinavir and ritonavir. *J. Med. Chem.* **2009**, *52*, 2964–2970.
- (62) ClinicalTrials.gov. Identifier NCT01927965.
- (63) Yang, D.; Ye, X. Y.; Xu, M. Enantioselective total synthesis of (–)-triptolide, (–)-triptonide, (+)-triptophenolide, and (+)-triptoquinone. *J. Org. Chem.* **2000**, *65*, 2208–2217.