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Synthesis and biological activity of acetyl-protected hydroxybenzyl diethyl phosphates (EHBP) as potential chemotherapeutic agents

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ABSTRACT

Several acetyl-protected hydroxybenzyl diethyl phosphates (EHBPs) that are capable of forming quinone methide intermediates were synthesized and their cell growth inhibitory properties were evaluated in four different human cancer cell lines. Compounds **1**, **1a**, and **1b**, corresponding to (4-acetyloxybenzyl diethylphosphate), (3-methyl-4-acetyloxybenzyl diethylphosphate), and (3-chloro-4-acetyloxybenzyl diethylphosphate), were significantly more potent than compounds **2** and **3**, (2-acetyloxybenzyl diethylphosphate) and (3-acetyloxybenzyl diethylphosphate), respectively. Using HT-29 human colon cancer cells, compounds **1** and **3** increased apoptosis, inhibited proliferation, and caused a G_2/M block in the cell cycle. Our data suggest that these compounds merit further investigation as potential anti-cancer agents.

Cancer is a major health problem world wide, accounting for significant morbidity and mortality.[1] For many cancers, currently available treatments are either palliative or ineffective. Thus, novel therapies are urgently needed either to prevent the development of cancer or; in the case where neoplasia has been initiated, to render the host organism cancer-free.[2] Partial reduction of a cancer burden is still worthwhile if an agent can significantly facilitate the use of concomitant therapies.[3]

Over thirty epidemiological studies, collectively describing results on greater than one million subjects, have established NSAIDs as the prototypical chemopreventive agents against many forms of cancer.[3] However, the use of NSAIDs is limited by their significant side effects, which includes gastrointestinal, renal, hypersensitivity reactions, and salicylate intoxication.[3] The gastric damage is primarily caused by breakdown of mucosal defense mechanisms (decreases in blood flow, bicarbonate, and mucus secretions) due to reductions in mucosal prostaglandin (PG) synthesis.[4] Nitric oxide–donating NSAIDs are a novel emerging class of anticancer compounds comprising an NSAID, an NO-releasing moiety and a spacer linking them. Their development was based on the observation that NO has some of the same properties as PGs within the gastric mucosa. For example, NO increases blood flow, reducing the effects of luminal irritants, it also increases mucus and bicarbonate secretions thus modulating other components of the mucosal defense systems.[5] Therefore, coupling an NO-releasing moiety to an NSAID might deliver NO to the site of NSAID-induced damage, thereby decreasing gastric toxicity. This rational proved to be sound as evidenced by animal[6,7] and human studies.[8,9] Many in vitro and animal studies also showed that NO–NSAIDs in general and NO-aspirin in particular were promising agents in cancer prevention/treatment,[10–13] with NO release being pivotal for the observed effects.[14] However, careful reexamination regarding the contribution to the overall biological effect of each of the three structural components of NO-aspirin in which the spacer joining the aspirin to the NO-releasing moiety was aromatic, led to the surprising conclusion that the NO-releasing moiety was not required for the observed biological effects. Rather, the spacer was responsible for the observed biological actions of NO-aspirin. The NO-releasing moiety acted as a leaving group to facilitate the release and activation of the spacer to a quinone methide (QM) intermediate that acted as powerful electrophile.[15–17] On this basis, here we report the synthesis and biological activity of a series of *o*-, *p*-, and *m*-acetyl-protected hydroxybenzyl diethyl phosphates (EHBPs) in which the nitrate leaving group is replaced by a substituted phosphate and the aspirin is replaced by an acetate. Electron-donating/withdrawing groups are also incorporated around the spacer to evaluate their effect on QM formation/stability and biological activity.

Several EHBPs were prepared as described in Scheme 1. Unsubstituted compounds **1**, **2**, and **3** were prepared from their respective hydroxybenzyl alcohols.[18,19] The phenolic part was selectively acylated with acetyl chloride[20] in the presence of triethyl amine

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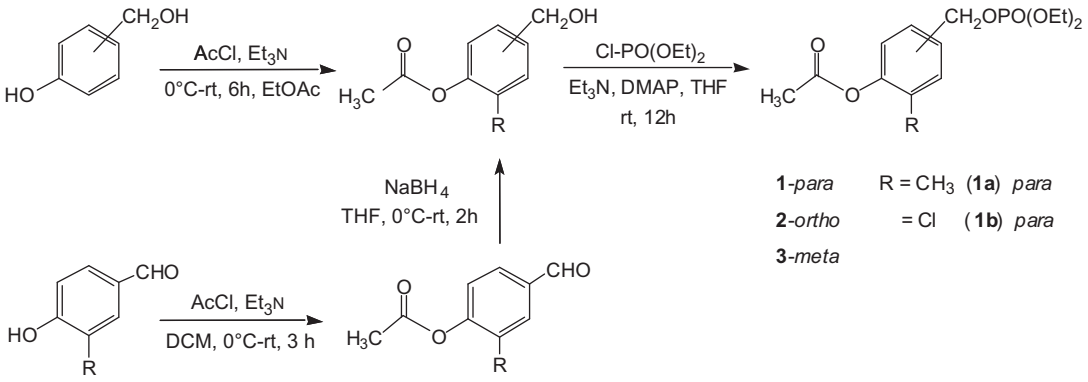

Scheme 1. Synthesis of EHBPs **1**, **1a**, **1b**, **2** and **3**.

and the resulting benzyl alcohol was treated with diethyl chloro phosphate in THF, yielding the desired acetyloxy benzyl diethyl phosphates.[21] For the substituted compounds **1a** and **1b**, the appropriate hydroxy benzaldehyde was acylated with acetyl chloride and triethyl amine as base; the resultant aldehyde was reduced with $NaBH_4$ to afford the corresponding alcohol. The benzyl alcohol thus obtained was phosphorylated with diethyl chloro phosphate to yield the desired phosphate derivatives.[22]

The major requirement for generating an *ortho* or *para* quinone methide (QM) is a protected hydroxy benzyl moiety to which a good leaving group is attached. Quinone methides in general are a highly reactive electrophilic species that can react with cellular nucleophiles such as GSH and DNA thus producing cytotoxic effects.[23,24] To study the reactivity of QMs, we have used a methyl moiety as an electron-donating group, and a chloro as an electron-withdrawing group in order to evaluate the effect of substitution in the benzyl moiety on the formation and hence the biological activity of QM.

We evaluated the inhibitory effects of agents **1**, **1a**, **1b**, **2**, and **3** on cell growth by an MTT assay using various human cancer cell lines.[25] Figure 1A shows growth inhibition profile of **1** in four different cancer cell lines of different tissue origin, that being of, colon, breast, pancreas, and leukemia. This data strongly suggests that the effects on cell growth inhibition are tissue type independent. The effects of **1**, **1a**, **1b**, **2**, and **3** on colon cancer cell growth are shown in Figure 1B. The data show that **1**, **1a**, and **1b**, are orders of magnitude more potent than **2** and **3**. From such dose–response curves we calculated the IC_{50}s for cell growth inhibition in different cancer cell lines (Table 1). The data clearly show that **1** is significantly more potent than **2** and **3** in all cell lines. The IC_{50}s for cell growth inhibition being 0.6–0.8 μM for **1**, 62–155 μM for **2**, and 162–451 μM for **3**. It should be noted that we had expected the IC_{50}s for cell growth inhibition for **1** and **2** to be similar as they are for *p*- and *o*-NO-ASA.[14] We believe that **2** did not behave as expected presumably because of steric interference between the diethyl phosphate and the acetyloxy groups making it difficult for the QM to form. It is noteworthy that **1** is in the order of 17–95-fold more potent than *p*-NO-ASA which has an IC_{50} for cell growth inhibition of 10–57 μM in various cancer cell lines.[10,11,26] Agent **3** acted as expected in having a very high IC_{50} for cell growth inhibition in all cancer cell lines, ranging from 162, 214, 273, and 451 μM in pancreas, breast, colon, and leukemia cells, respectively. This agent is comparable to *m*-NO-ASA in terms of its IC_{50} for cell growth inhibition, both of which are not capable of forming a

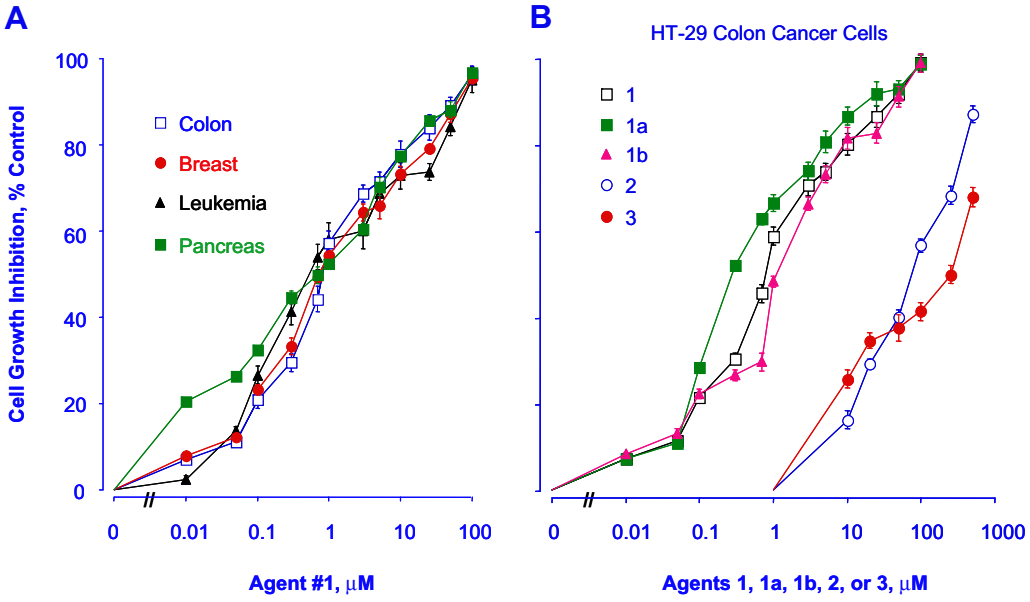

Figure 1. Effect of EHBPs on cell growth. Panel A: Cancer cells of different tissue origin were treated with different concentration of **1** for 24 h. Panel B: HT-29 human colon cancer cells were treated with different concentrations of **1**, **1a**, **1b**, **2** and **3**. From such studies the IC_{50}s for cell growth inhibition were determined, Table 1. Results are mean ± SEM of three different experiments performed in triplicate.

Table 1
IC_{50} values at 24 h for cell growth inhibition in different cancer cell lines.

Compounds	Origin/Cell line, IC_{50}, μM			
	Colon HT-29	Leukemia Jurkats	Breast MCF-7	Pancreas BxPC-3
1	0.82 ± 0.07*	0.65 ± 0.03*	0.67 ± 0.02*	0.67 ± 0.02*
1a	0.31 ± 0.02*	0.29 ± 0.06*	0.32 ± 0.01*	0.28 ± 0.01*
1b	1.3 ± 0.24*	0.68 ± 0.04*	0.70 ± 0.02*	0.65 ± 0.03*
2	82.7 ± 1.8	110 ± 2	62.7 ± 3.4	155 ± 5
3	273 ± 5	451 ± 9	214 ± 4	162 ± 3

Colon, leukemia, breast, and pancreas cancer cell lines were treated with various concentrations of **1**, **1a**, **1b**, **2**, and **3** as described under Experimental Section/Biology. Cell numbers were determined at 24 h from which IC_{50} values were calculated. Results are mean ± SEM. of five to seven different experiments done in triplicate.
* P <0.001 compared to **2** and **3**.

QM intermediate. We also evaluated the effects of electron-donating and electron-withdrawing groups about the benzyl moiety to determine their effect on the cell growth inhibitory activity of compound **1**. Incorporation of a methyl group about the benzyl moiety (compound **1a**) increases the potency between 2.1- and 2.6-fold in all the cell lines studied here (Table 1, ratios of **1** to **1a**). This enhanced potency may be ascribed to the ease by which the QM intermediate is formed. On the other hand, substitution with a chlorine atom into the benzyl moiety (compound **1b**), did not have an effect on the potency in three of the cell lines studied (Table 1, ratios of **1** to **1b**). In HT-29 colon cancer cells, **1b** was less potent by about 1.5-fold (Table 1 and Fig 1B).

Two determinants of cellular mass are proliferation and apoptosis. We examined the effects of different concentrations of **1** and **3** after 24 h of treatment on these two parameters using HT-29 colon cancer cells.[27] Figure 2A–C shows that the percentage of apoptotic cells increased in a concentration dependent manner: for **1** these ranged from 25 ± 2% at 10 μM to 79 ± 4% at 50 μM. At the same time, the proliferation marker, PCNA, decreased in a dose-dependent manner. From Figure 2B we can discern that inhibition of proliferation at low concentrations contributed more than apoptosis to the inhibitory effects of **1**. For **3**, apoptosis increased from 19 ± 1% at 100 μM to 79 ± 2% at 300 μM. Also, PCNA, decreased in a dose-dependent manner. However, it appears that for this agent

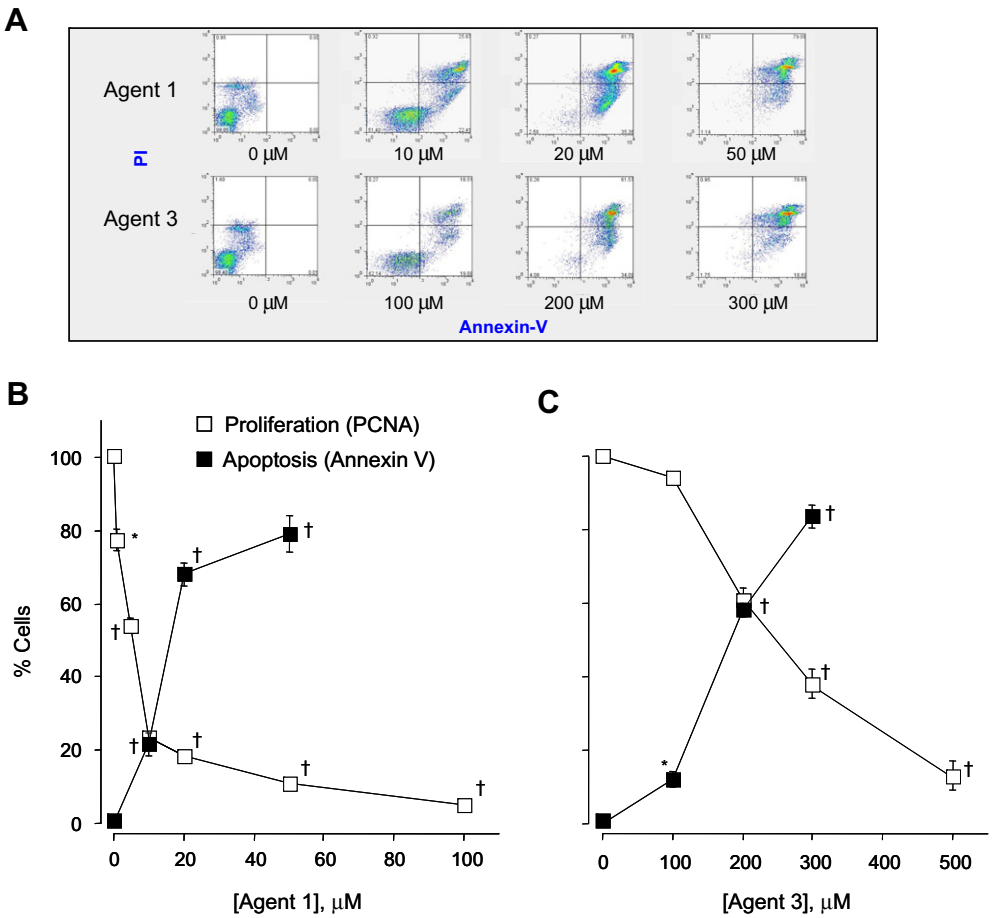

Figure 2. Compounds **1** and **3** induce apoptosis and inhibit proliferation in HT-29 colon cancer cells. Cells were treated with **1** and **3** at the concentration indicated for 24 hr after which the cells were stained with Annexin V and propidium iodide and subjected to flow cytometric analysis (Panel A). The percentage of apoptotic cells increased in a concentration dependent manner. Also, cells were treated with different concentrations of **1** and **3** for 24 h after which PCNA expression was determined by flow cytometry and expressed as percentage positive cells. Results for **1** and **3** are shown in panels B and C, respectively. Results are mean ± SEM of three different experiments. *P <0.05; †P <0.01 compared with untreated cells.

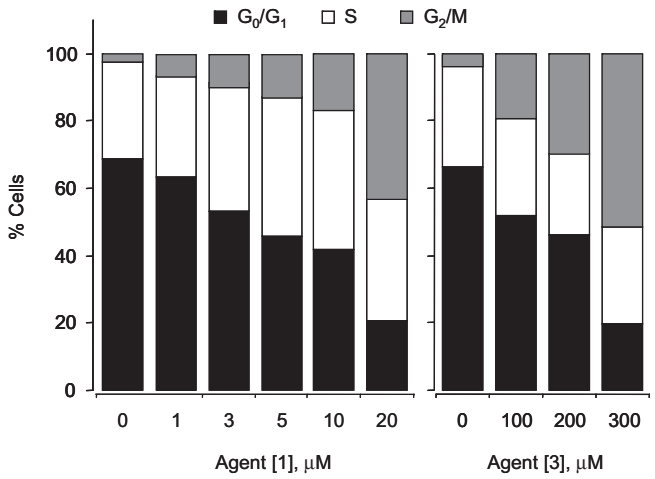

Figure 3. Effect of **1** and **3** on cell cycle in HT-29 colon cancer cells. Cells were treated for 24 h with various concentrations of **1** and **3**, and their cell cycle phase distribution was determined by flow cytometry. Results are representative of two different experiments. This study was repeated twice generating results within 10% of those presented here.

apoptosis and inhibition of proliferation contribute more or less equally to the inhibitory effects of **3** (Fig. 2C).

We examined whether cell growth inhibition by **1** and **3** were attributable to changes in the phases of the cell cycle. HT-29 cells were treated with different concentrations of **1** and **3** for 24 h after which the population of cells were examined. Since these compounds have different potency ratios, it was of interest to examine them at a time point where they have differential effects on proliferation and apoptosis. Therefore, 24 h was chosen as a representative time point in this study. For both **1** and **3**, there was a dose-dependent reduction in the population of cells in G_0/G_1, the S phase increased at low concentrations but was essentially unchanged at higher concentrations, and the G_2/M population increased (Fig. 3). For example, treatment with 3 μM of **1**, caused a reduction in the population of cells in G_0/G_1 phase from 74.8% to 53.2% and cells in the S phase increased from 19.4% to 38.2%, and G_2/M increased from 5.8% to 9.6%. At 10 μM of **1**, G_0/G_1 was reduced to 42.9%, S phase was 40.8%, and G_2/M population had increased to 16.3%. These data are consistent with a G_2/M block as has been reported for other compounds in different cell lines.[26,28,29] Agent **3** behaved similarly to **1** but at much higher concentrations, 100–300 μM (Fig. 3).

In summary, the antiproliferative activity of EHBPs against four human cancer cell lines was evaluated. Compounds **1**, **1a**, and **1b** showed strong cell growth inhibition in all human cancer cell lines at low micromolar concentrations. Compounds **2** and **3** were significantly less potent than **1** in these cell lines. Compounds **1** and **3** increased apoptosis and inhibited cell proliferation in a time-dependent manner. This was accompanied by changes in the cell cycle, showing a G_2/M arrest. Further studies are needed to evaluate the anticarcinogenic properties of these compounds and their molecular targets.

Acknowledgment

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18. *General:* All moisture sensitive reactions were performed under inert atmosphere (argon) using syringe–septum cap techniques. All glassware was oven-dried. Freshly distilled anhydrous solvents were used for all reactions. THF was distilled from sodium/benzophenone, while DCM was distilled from calcium hydride. The ^{1}H NMR high resolution spectra were recorded using Varian Inova 500 MHz (^{1}H NMR) spectrometers. All spectra were recorded in $CDCl_3$. Splitting patterns are described as singlet (s), doublet (d), doublet of doublet (dd), triplet (t), quartet (q), and broad (br). The value of chemical shifts (δ) are given in ppm relative to TMS as internal standard and coupling constants (J) are given in hertz (Hz). Thin-layer chromatography was performed on 250 μ silica plates (Analtech) and column chromatographic purifications were performed on 100–200 mesh silica gel (Natland). Unless otherwise noted, all the aldehydes and reagents herein used were acquired from Sigma–Aldrich and Across Chemicals and used without further purification. Extracts were dried over anhydrous Na_2SO_4 and filtered prior to removal of all volatiles under reduced pressure. The mass spectra were recorded on a JEOL JMS-SX 10217 instrument (EI).
19. *4-Acetyloxybenzyl diethyl phosphate* (**1**): To a solution of *p*-acetoxybenzyl alcohol (1 g, 6.0 mmol) in dry THF (50 ml) was added triethyl amine (Et_3N, 1.25 ml, 9.0 mmol), DMAP (75 mg, 0.6 mmol) and diethyl chlorophosphate (0.95 ml, 6.6 mmol) over a 30 min period at room temperature under an argon atmosphere. The resultant white heterogeneous mixture was stirred for 12 h. After completion of the reaction as monitored by TLC, water was added and the mixture was extracted using EtOAc. The organic layer was then separated and washed with aq $NaHCO_3$ and brine. The combined organic layers were dried and concentrated under vacuo. The residue was purified by silica gel column chromatography, eluting with 40% EtOAc in hexane to afford compound 1 (1.4 g, 82% yield). ^{1}H NMR ($CDCl_3$, 500 MHz): δ_H 7.42 (d, J = 8.0 Hz, 2H), 7.10 (d, J = 8.0 Hz, 2H), 5.05 (d, J = 8.0 Hz, 2H), 4.05–4.12 (m, 4H), 2.31 (s, 3H), 1.31 (t, J = 7.0 Hz, 6H). ^{13}C NMR ($CDCl_3$, 125 MHz): δ_C 16.11 (d, J_{CP} = 6.8 Hz), 21.0, 63.91 (d, J_{CP} = 6.1 Hz), 68.39 (d, J_{CP} = 5.3 Hz), 121.88, 129.18, 133.74 (d, J_{CP} = 6.8 Hz), 150.86, 169.30. ^{31}P NMR ($CDCl_3$, 202 MHz): δ_P −0.35. ESIMS: m/z 303 (M^++1), 325 (M^++Na).
2-Acetyloxybenzyl diethyl phosphate (**2**): The title compound was obtained from 2-acetoxybenzyl alcohol using the above procedure giving a 65% yield. ^{1}H NMR ($CDCl_3$, 500 MHz): δ_H 7.41 (d, J = 8.0 Hz, 1H), 7.40 (d, J = 7.6 Hz, 1H), 7.32 (t, J = 7.0 Hz, 1H), 7.18 (t, J = 7.0 Hz, 1H) 5.19 (s, 2H), 4.20 (m, 4H), 2.10 (s, 3H), 1.36 (t, J = 7.0 Hz, 6H). ^{13}C NMR ($CDCl_3$, 125 MHz): δ_C 15.76 (d, J_{CP} = 6.1 Hz), 20.63, 60.98, 64.48 (d, J_{CP} = 6.1 Hz), 119.52, 124.72, 126.66 (d, J_{CP} = 7.6 Hz), 129.40, 129.86, 148.60, 170.43. ^{31}P NMR ($CDCl_3$, 202 MHz): δ_P −5.81. ESIMS: m/z 303 (M^++1), 349 (M^++2Na).
3-Acetyloxybenzyl diethyl phosphate (**3**): The title compound was obtained from 3-acetoxybenzyl alcohol using the procedure described for compound **1**. 85% yield, ^{1}H NMR ($CDCl_3$, 500 MHz): δ_H 7.38 (t, J = 7.6 Hz, 1H), 7.25 (d, J = 7.6 Hz, 1H), 7.14 (bs, 1H), 7.06 (dd, J = 7.6, 1.95 Hz, 1H), 5.06 (d, J = 8.2 Hz, 2H), 4.05–4.12 (m, 4H), 2.30 (s, 3H), 1.31 (t, J = 6.5 Hz, 6H). ^{13}C NMR ($CDCl_3$, 125 MHz): δ_C 15.67 (d, J_{CP} = 6.1 Hz), 20.67, 63.51 (d, J_{CP} = 5.3 Hz), 67.82, 120.53, 121.27, 124.61, 129.23, 137.39 (d, J_{CP} = 6.8 Hz), 150.44, 168.91. ^{31}P NMR ($CDCl_3$, 202 MHz): δ_P −0.46. ESIMS: m/z 303 (M^++1).
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22. *4-(Acetyloxy)-3-methylbenzyl diethyl phosphate* (**1a**): The title compound was obtained from 4-(acetoxy)-3-methylbenzyl alcohol using the procedure described for compound **1**. The yield was 68%. ^{1}H NMR ($CDCl_3$, 500 MHz): δ_H 7.27 (s, 1H), 7.24 (d, J = 8.5 Hz, 1H), 7.01 (d, J = 8.5 Hz, 1H), 5.02 (d, J = 5.0, 2H), 4.07–4.10 (m, 4H), 2.32 (s, 3H), 2.19 (s, 3H), 1.31 (t, J = 7.0 Hz, 6H). ^{13}C NMR ($CDCl_3$, 125 MHz): δ_C 15.74 (d, J_{CP} = 6.8 Hz), 15.83, 20.47, 63.58 (d, J_{CP} = 5.3 Hz), 68.15, 121.79, 126.26, 130.14, 130.45, 133.43 (d, J_{CP} = 6.8 Hz), 149.07, 168.85. ^{31}P NMR ($CDCl_3$, 202 MHz): δ_P −0.47. ESIMS: m/z 317 (M^++1), 339 (M^++Na).
4-(Acetyloxy)-3-chlorobenzyl diethylphosphate (**1b**): The title compound was obtained from 4-(acetoxy)-3-chlorobenzyl alcohol using the procedure

described for compound **1**. The yield was 72%. ^{1}H NMR ($CDCl_3$, 500 MHz): δ_H 7.49 (d, J = 1.95 Hz, 1H), 7.28 (dd, J = 8.5 Hz, 1.95 Hz, 1H), 7.14 (d, J = 8.5 Hz, 1H), 5.02 (d, J = 5.0, 2H), 4.07–4.13 (m, 4H), 2.35 (s, 3H), 1.32 (t, J = 7.0 Hz, 6H). ^{13}C NMR ($CDCl_3$, 125 MHz): δ_c 15.70, 20.16, 63.77, 67.26, 116.11, 123.52, 126.77, 129.14, 134.94 (d, J_{CP} = 6.1 Hz), 146.59, 167.96. ^{31}P NMR ($CDCl_3$, 202 MHz): δ_p −0.70. ESIMS: m/z 337 (M^++1), 339 (M^++1), 359 (M^++Na), 361 (M^++Na).

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25. *Cell culture:* Four human cancer cell lines, HT-29 (colon), Jurkat T (leukemia), BxPC-3 (pancreas) and MCF-7 (breast), purchased from American Type Culture Collection (Manassas, VA), were maintained in RPMI 1640 (HT-29 and Jurkat) or DMEM (BxPC-3 and MCF-7) supplemented with 10% fetal bovine serum and 50 U/mL penicillin G and 50 μg/mL streptomycin sulfate (Gibco, BRL) at 37 °C in a humidified atmosphere containing 5% CO_2. All compounds were dissolved in DMSO (100 mM) and diluted to the desired concentrations. The final concentration of DMSO was adjusted to 1% in all media.
Cell growth inhibition assay: A commercially available kit (colorimetric MTT assay kit Roche; Indianapolis, IN) was used to estimate proliferation according to the manufacturer's instruction. Cells were seeded in a 96-well plates at the cell density of 10×10^3 cells/well and allowed to adhere or grow overnight. Compounds **1**, **1a**, **1b**, **2** and **3** were added at indicated concentrations to the culture media and the cells were incubated for 24 h. Viable cells were quantified with MTT substrate according to the manufacturer's instructions. Growth inhibition was expressed as percentage of the corresponding control.
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27. *Cell proliferation and cell cycle analysis:* HT-29 cells (1×10^6 cells/mL) were treated for 24 h with various concentrations of **1** and **3**. PCNA was determined using an ELISA Kit (Calbiochem, La Jolla, CA), in accordance with the manufacturers protocol. For cell cycle analysis, HT-29 cells (0.5×10^6) were treated with **1** and **3** for 24 h. Cells were harvested by trypsinization and centrifugation. They were then fixed in 70% ethanol for 10 min at −20 °C, pelleted (5000 rpm × 10 min at 4 °C), resuspended and incubated in PBS containing 1% FBS/0.5% NP-40 on ice for 5 min. Cell were washed again in 500 μL of PBS/1% FBS containing 40 μg/mL propidium iodide (used to stain for DNA) and 200 μg/mL RNase type IIA, and analyzed within 30 min by flow cytometry. Cell cycle phase distributions of control and treated HT-29 cells were obtained using a Coulter Profile XL equipped with a single argon ion laser. For each subset, >10,000 events were analyzed. All parameters were collected in list mode files. Data were analyzed on a Coulter XL Elite Work station using the Software programs Multigraph™ and Multicycle™. The percentage of cells in G_0/G_1, G_2/M, and S phases was determined form DNA content histograms.
Determination of apoptosis: HT-29 cells (0.5×10^6 cells/mL) were treated for 24 h with various concentrations of **1** and **3**. Cells were washed with and resuspended in 1× Binding Buffer (Annexin V binding buffer, 0.1 M Hepes/NaOH (pH 7.4), 1.4 M NaCl, 25 mM $CaCl_2$) from BD BioSciences (San Diego, CA). Then 5 μL of Annexin V-FITC (final concentration: 0.5 μg/ml) was added followed by Propidium iodide (final concentration 20 μg/mL). The cells were incubated at room temperature for 5–15 min in the dark. Finally, the cells were transferred to FACS tubes and analyzed by flow cytometry as described above using Flow Jo software.
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