Isosteric Phosphonate Analogs of ET-16-OMe. Synthesis and Biological Evaluation of the Enantiomers of 2′-(Trimethylammonio)ethyl 4-(Hexadecyloxy)-3-methoxybutanephosphonate and 2′-(Trimethylammonio)ethyl 4-(Hexadecylthio)-3-methoxybutanephosphonate

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The enantiomers of two isosteric phosphonate analogs of the ether-linked antitumor agent 1-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine (ET-18-OMe) were synthesized and evaluated for their cytotoxicity against various mouse leukemic cell lines in vitro and in vivo. The key step in the synthesis of the alkyloxy and alkylthio analogs (**1** and **2**, respectively) is the opening of an epoxide [hexadecyl 2-oxiranylmethyl ether (**4**) or hexadecyl 2-oxiranylmethyl thioether (**8**)] by $LiCH_2P(O)(OMe)_2$ using $BF_3{\cdot}Et_2O$ in tetrahydrofuran at low temperature. The cytotoxic activities of the hexadecyloxy and hexadecylthio phosphonate analogs of ET-18-OMe (**1** and **2**) against the murine leukemias WEHI-3B, L1210, and P388 were similar, indicating that substitution of a sulfur atom for oxygen in the long-chain ether does not result in a significant difference in cytotoxicity. The IC_{50} values of **1** and **2** were in the range of 1–5 μM. Alkyloxy phosphonate **1** was highly effective in inhibiting the growth of WEHI-3B and P388 tumors implanted in BALB/C mice. The alkyloxy and alkylthio phosphonates **1** and **2** prolonged the survival of CD1 mice bearing L1210 tumors. The antitumor activities of the phosphonate analogs of ET-18-OMe in these in vitro and in vivo studies were independent of chirality, consistent with previous studies with the enantiomers of 1-*O*-hexadecyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine.

Introduction

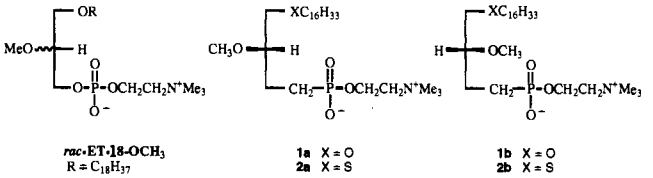
We recently communicated[1] a new method for preparing isosteric phosphonocholine (*S*)-**1a** and showed that this compound was as effective against WEHI-3B cells both in vitro and in vivo as the well-known ether lipid *rac*-ET-18-OMe and its hexadecyl analog 1-*O*-hexadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (*rac*-ET-16-OMe) (see Chart 1 for structures), which are generally used as the yardstick for comparison of the cytotoxic activity of ether-linked lipids.[2] Phosphonocholines **1** and **2** have a methylene group substituted for the oxygen atom joining the phosphorus atom to the glycerol moiety. Unlike ET-18-OMe, **1** and **2** are resistant to hydrolysis catalyzed by phospholipase C, and thus these analogs may be long-acting antineoplastic agents. Analogs of *rac*-ET-18-OMe that contain a 1-alkylthio group in place of the 1-alkyloxy group have been found to possess antineoplastic activity against various cells that is similar to[3] or even higher than[4] that of *rac*-ET-18-OMe. The present study reports the synthesis of the enantiomers of the hexadecyloxy and hexadecylthio phosphonates **1** and **2**, respectively, and compares the activities of these compounds against leukemic WEHI-3B and L1210 cells, as well as P388 and adriamycin-resistant P388 (P388-ADR) cells in culture and in vivo.

Chemistry

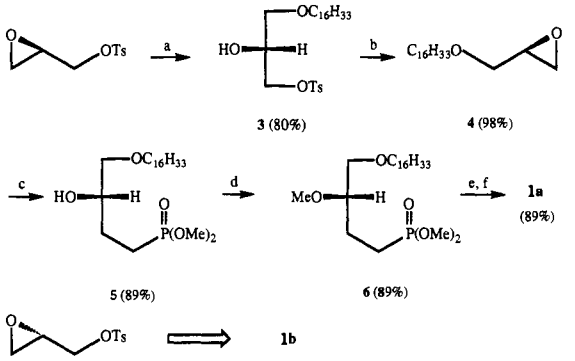
Alkyloxy Phosphonate (1). Phosphonolipid **1a** was prepared as outlined in our earlier communication.[1] The key step in the synthetic sequence (Scheme 1) is the ring-opening reaction of oxirane **4** with lithium dimethyl methanephosphonate in the presence of $BF_3{\cdot}Et_2O$ at low temperature.

Alkylthio Phosphonate (2). Hexadecylthio phosphonolipid **2a** was prepared in a sequence that utilized 1-(hexadecylthio)-*sn*-glycerol (**7a**) as the starting material (Scheme 2). The latter compound was prepared by in situ $NaBH_4$-mediated opening of (*S*)-glycidol [prepared by asymmetric epoxidation of allyl alcohol] with hexadecyl

Chart 1



Scheme 1.[a] Synthesis of (Hexadecyloxy)methoxyphosphonocholines 1a and 1b



[a] Reagents: (a) $BF_3{\cdot}Et_2O$, $C_{16}H_{33}OH$; (b) K_2CO_3, MeOH; (c) $LiCH_2P(O)(OMe)_2$, $BF_3{\cdot}Et_2O$, THF, −78 to −20 °C; (d) CH_2N_2, SiO_2, 0 °C or MeOTf, 2,6-di-*t*-C_4H_9-4-Me-Py, CH_2Cl_2, reflux; (e) (i) Me_3SiBr, CH_2Cl_2; (ii) THF–H_2O (8:1), 2 h, room temperature (f) choline tosylate, Cl_3CCN, py, 50 °C, 48 h.

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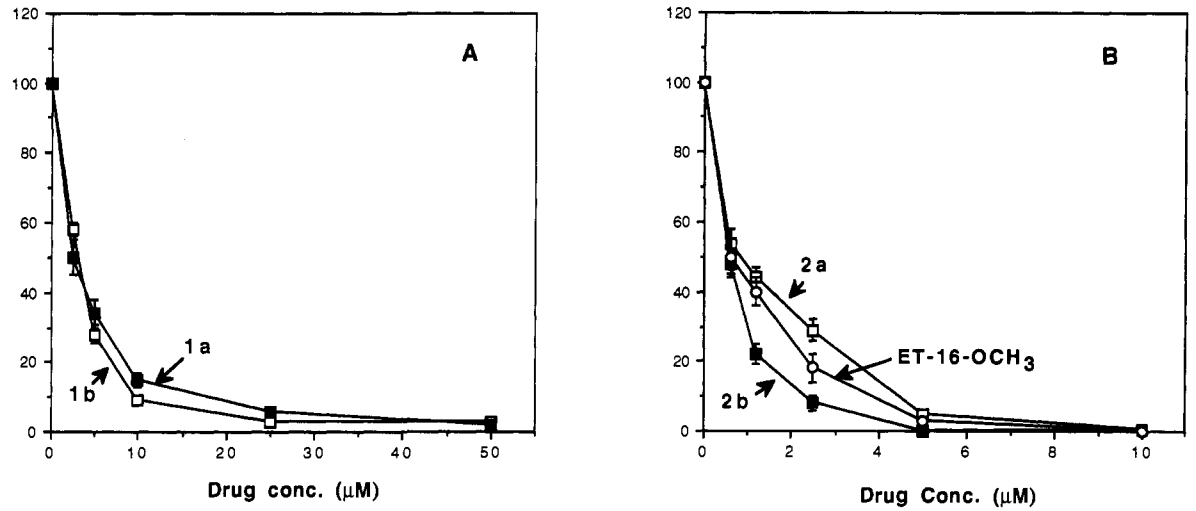
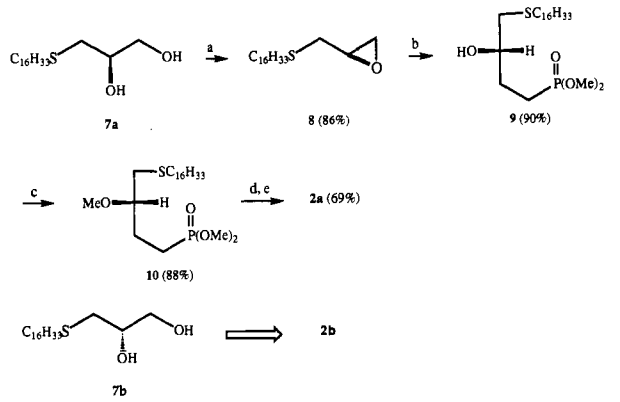


Figure 1. Effects of compounds **1a** and **1b** (A) and compounds **2a**, **2b**, and *rac*-ET-16-OMe (B) on the growth of WEHI-3B cells in soft agar. Clonogenic assays were performed as described in the Experimental Section, and drugs were added to the medium when the cells were cultured. Values represent the mean ± SD (n = 5).

Scheme 2.[a] Synthesis of (Hexadecylthio)methoxyphosphonocholines 2a and 2b



[a] Reagents (a) Ph_3P, $EtO_2CN{=}NCO_2Et$, C_6H_6; (b) $LiCH_2P(O)(OMe)_2$, $BF_3{\cdot}Et_2O$, THF, −78 to −20 °C; (c) CH_2N_2, SiO_2, 0 °C; (d) (i) Me_3SiBr, CH_2Cl_2; (ii) THF–H_2O (8:1), 2 h, room temperature; (e) choline tosylate, Cl_3CCN, py, 50 °C, 48 h.

mercaptan.[5] 3-(Hexadecylthio)-*sn*-glycerol (**7b**), which was prepared in the analogous in situ $NaBH_4$-mediated ring-opening reaction of (*R*)-glycidol, was used as the starting material for the preparation of **2b**. Oxirane **8** was obtained in 86% yield from diol **7** by using the Mitsunobu reaction. Ring opening of oxirane **8** with lithium dimethyl methanephosphonate, as in Scheme 1, proceeded in high yield. Subsequent methylation with diazomethane in ether in the presence of activated silica gel at 0 °C, followed by hydrolysis of the methyl ester groups of phosphonate ester **10** and coupling of the derived phosphonic acid with choline tosylate, produced the required phosphonocholines **2a** and **2b**.

Biological Results and Discussion

Inhibition of WEHI-3B Growth in Cell Culture. Figure 1A shows that the (*S*) and (*R*) stereoisomers of **1** were equally effective in inhibiting the growth of the monocytic leukemic cell line WEHI-3B, as estimated using a clonogenic assay. Figure 1B shows that the enantiomers of (hexadecylthio)phosphonocholine **2** also had a similar potency against WEHI-3B cells and were as effective as ET-16-OMe. We reported recently that a nonisosteric

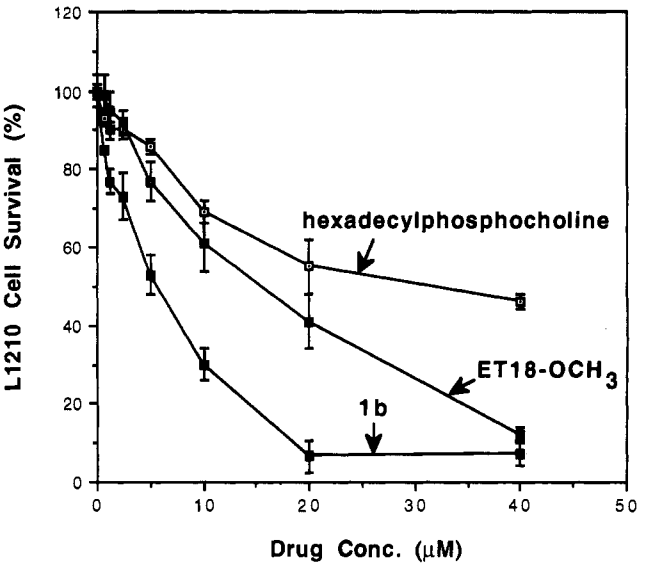


Figure 2. Comparison of the effects of compound **1b**, *rac*-ET-18-OMe, and hexadecylphosphocholine on the survival of leukemic L1210 cells in vitro. The drugs were added to the medium when the cells were cultured. Cell survival was determined by counting the number of viable cells at the conclusion of the experiment using the trypan blue dye exclusion assay.

phosphonate analog of ET-18-OMe was slightly less toxic than ET-16-OMe against the human leukemic cell line HL-60 and the human undifferentiated cervical carcinoma C-41.[6] The isosteric phosphonate **1a** was equipotent with ET-16-OMe against WEHI-3B growth.[1]

Inhibition of L1210 Growth in Cell Culture. Figure 2 presents the results of a comparison of the cytotoxicities of **1b** and two ether lipids currently in clinical trials, i.e., hexadecylphosphocholine[7] and *rac*-ET-18-OMe. We observed that **1b** was more effective in inducing 50% inhibition of L1210 cell death in vitro than ET-18-OMe and hexadecylphosphocholine by factors of about 2 and 20, respectively. The 50% inhibition dosage (IC_{50} value), estimated by clonogenic assay, for L1210 growth by **1a** was about 2.5 μM.

Inhibition of P388 Growth in Cell Culture. We also studied the effects of phosphonate **1** on the growth of P388 cells. The compound was equally active against adriamycin-sensitive and resistant cells, with an IC_{50} value for

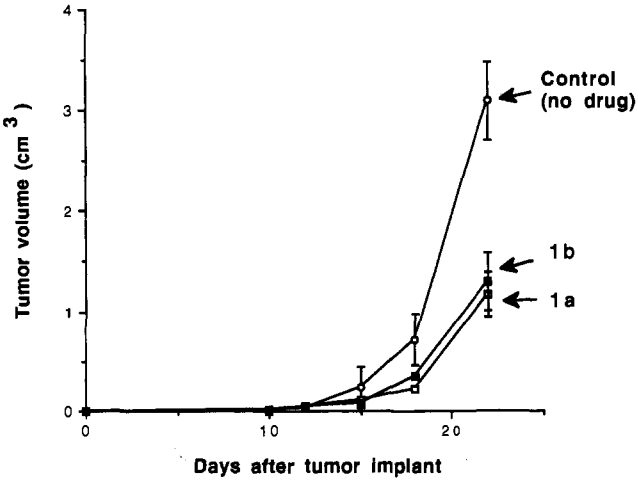


Figure 3. Effects of compounds **1a** and **1b** on the growth of WEHI-3B cells injected subcutaneously in BALB/C mice, compared with normal growth of cells in control (untreated) animals. Drug treatment (5 mg/kg per day, 5 days per week, intramuscularly) was initiated 3 days after injection of WEHI-3B cells. Data are the mean ± SD (n = 5).

both P388 and P388-ADR cells of approximately 2.5 μM (data not shown).

In Vivo Studies. The cytotoxicities of the enantiomers of the phosphonocholines **1** and **2** were evaluated by measuring the delay of tumor growth developed subcutaneously in BALB/C mice. Figure 3 shows that WEHI-3B tumor growth was suppressed by about 50–60% after 3 weeks when **1a** or **1b** was administered at a dose of 5 mg/kg. As noted in the in vitro studies, the enantiomers displayed no difference in cytotoxicity, indicating that the biological activity is independent of the stereochemistry. Figure 4A shows that **1a** and **1b** also delayed the growth of P388 cells in vivo with equal potency, as estimated from suppression of tumor volume; furthermore, these phosphonolipids were effective against the growth of P388-ADR cells, which is an adriamycin-resistant cell line (Figure 4B). Figure 5 shows that **1a**, **1b**, **2a**, and **2b** were highly effective in inhibiting L1210-mediated death of CD1 mice and were slightly more effective than *rac*-ET-16-OMe. Thus phosphonocholines **1** and **2** are promising candidates as new antitumor agents.

Conclusions

We have found that alkyloxy- and alkylthiophosphonocholines **1** and **2** have potent cytotoxicities toward WEHI-3B, P388, and L1210 murine leukemias in cell culture and in vivo. The absence of a significant dependence of biological activity on the stereochemistry of **1** and **2** is consistent with the finding that the in vitro cytotoxicity of ET-16-OMe does not depend on chirality.[8] The substitution of a sulfur atom for an oxygen atom in the long-chain ether linkage does not appear to influence the cytotoxic activity. The unique structural feature in these antitumor ether phospholipids is the presence of a phosphono group, which makes **1** and **2** resistant to hydrolysis on the "glycerol" side of the molecule; thus, **1** and **2** may have an enhanced lifetime compared with ET-18-OMe in cells with high phospholipase C activity.

Experimental Section

Chemistry. ^{1}H NMR spectra were recorded on an IBM-Bruker 200-MHz spectrometer using Me_4Si as an internal standard. Infrared spectra were recorded on a Perkin-Elmer 1600 FT spectrophotometer. Optical rotations were measured on a JASCO Model DIP-140 digital polarimeter using a 1-dm cell. Microanalyses were performed by Desert Analytics (Tucson, AZ). Melting points were measured using a Thomas-Hoover apparatus and are uncorrected. The progress of reactions was monitored using glass-backed silica gel GF TLC plates (Analtech, Newark, DE), with 10% sulfuric acid solution in ethanol and/or short-wavelength ultraviolet light to visualize the spots. For flash chromatography and for the methylation reaction with diazomethane, E. Merck silica gel 60 (230–400 ASTM mesh, purchased from Aldrich Chemical Co.) was used. Methylene chloride and pyridine were distilled from calcium hydride and barium oxide, respectively. Methanol was refluxed and distilled over magnesium metal. THF was refluxed for several hours over sodium benzophenone ketyl before use. (*R*)- and (*S*)-glycidyl tosylates and 1-hexadecanol (purchased from Aldrich) were dried overnight under vacuum (0.1 mmHg) at room temperature before use. All other reagents were obtained from commercial sources and were used as received unless otherwise stated.

Synthesis of the Enantiomers of 2′-(Trimethylammonio)-ethyl 4-(Hexadecyloxy)-3-methoxybutanephosphonate (1a,b) (Scheme 1). 1-*O*-Hexadecyl-*sn*-glycerol 3-*O*-*p*-Toluenesulfonate (3). The reaction of 4.58 g (20 mmol) of (*R*)-(−)-glycidyl tosylate and 7.28 g (30 mmol) of 1-hexadecanol in 50 mL of methylene chloride in the presence of catalytic boron trifluoride etherate was carried out as described previously.[9] After purification by flash chromatography (elution with 5:1 hexane/ethyl acetate), followed by three recrystallizations from ether–hexane, there was obtained 7.41 g (80%) of ring-opened product **3**: mp 59.5–61.0 °C; R_f 0.30 (4:1 hexane/ethyl acetate); $[\alpha]^{25}_D$ −6.24° (c 5.0, C_6H_6); IR (KBr) 3600, 1360, 1182, 1130, 1102, 846, 821 cm^{-1}; NMR ($CDCl_3$) δ 7.82 (d, 2H, J = 8.5 Hz), 7.32 (d, 2H, J = 8.5 Hz), 4.05 (m, 3H), 3.44 (d, 2H, J = 5.0 Hz), 3.39 (t, 2H, J = 7.0 Hz), 2.45 (s, 3H), 2.15 (s, 1H), 1.4–1.6 (m, 2H), 1.29 (br s, 26H), 0.91 (br t, 3H, J = 6.7 Hz).

3-*O*-Hexadecyl-*sn*-glycerol 1-*O*-*p*-Toluenesulfonate. The enantiomer of **3** was prepared in 79% yield from (*S*)-(+)-glycidyl tosylate by the procedure described above; $[\alpha]^{25}_D$ +6.37° (c 5.0, C_6H_6).

Hexadecyl (*R*)-2-Oxiranylmethyl Ether (4). To a suspension of 7.10 g (15 mmol) of tosylate **3** in 100 mL of dry methanol was added 4.17 g (30 mmol) of powdered potassium carbonate at 0 °C. The reaction mixture was stirred for 3 h at 0 °C, diluted with 300 mL of ethyl ether, and filtered through a pad of silica gel. The filtrate was concentrated under reduced pressure, and the residue was dissolved in hexane and filtered through a pad of silica gel to give 3.95 g (98%) of epoxide **4** as a white solid, which was used without further purification: mp 34.5–35.5 °C; R_f 0.62 (9:1 hexane/ethyl acetate); $[\alpha]^{28}_D$ +8.70° (c 5.0, C_6H_6); IR (KBr) 1126, 858 cm^{-1}; NMR ($CDCl_3$) δ 3.74 and 3.68 (AB q, 1H, J = 11.5, 3.1 Hz), 3.6–3.3 (m, 3H), 3.2–3.1 (m, 1 H), 2.81 and 2.79 (AB q, 1H, J = 4.9, 4.2 Hz), 2.62 and 2.60 (AB q, 1H, J = 5.0, 2.7 Hz), 1.4–1.6 (m, 2H), 1.26 (br s, 26H), 0.88 (t, 3H, J = 6.7 Hz). Anal. Calcd for $C_{19}H_{38}O_2$: C, 76.45; H, 12.83. Found: C, 76.47; H, 12.71.

Hexadecyl (*S*)-2-Oxiranylmethyl Ether. The enantiomer of **4** was prepared in 97% yield from the tosylate by the procedure described above; $[\alpha]^{28}_D$ −8.91° (c 5.0, C_6H_6).

Dimethyl 4-(Hexadecyloxy)-3(*S*)-hydroxybutanephosphonate (5). To a solution of 4.97 g (40 mmol) of dimethyl methanephosphonate in 30 mL of dry THF was added dropwise 16 mL (40 mmol) of n-butyllithium (a 2.5 M solution in hexane), and the reaction mixture was stirred for 30 min at −78 °C. To this mixture was added dropwise boron trifluoride etherate (5 mL, 40 mmol), followed by a solution of 5.24 g (20 mmol) of epoxide **4** in 100 mL of THF. The reaction mixture was stirred for 3 h at −78 °C and then warmed to −20 °C and stirred for 1 h. The mixture was quenched by the addition of saturated aqueous ammonium chloride solution and was concentrated under reduced pressure. The product from the aqueous residue was extracted with ether, and the combined extracts were washed with brine, dried over sodium sulfate, and concentrated under vacuum. Purification by flash chromatography on silica gel (elution with chloroform–methanol, 25:1) gave 7.54 g (89%) of hydroxy phosphonate **5** as a white solid after lyophilization from hexane: mp 38.5–40.5 °C; R_f 0.41 (9:1 chloroform–methanol);

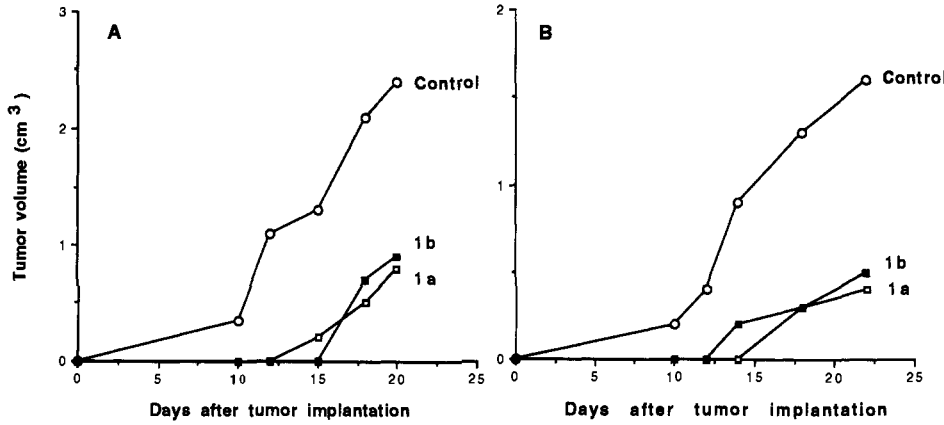


Figure 4. Effects of compounds **1a** and **1b** on the growth of ADR-sensitive P388 cells (A) and ADR-resistant P388 cells (B) injected subcutaneously in BALB/C mice. The results are compared with the normal growth of cells in control (untreated) animals. Drug treatment (5 mg/kg per day, 5 days per week, intramuscularly) was initiated 3 days after injection of P388 or P388-ADR cells. Data are the mean ± SD (n = 5).

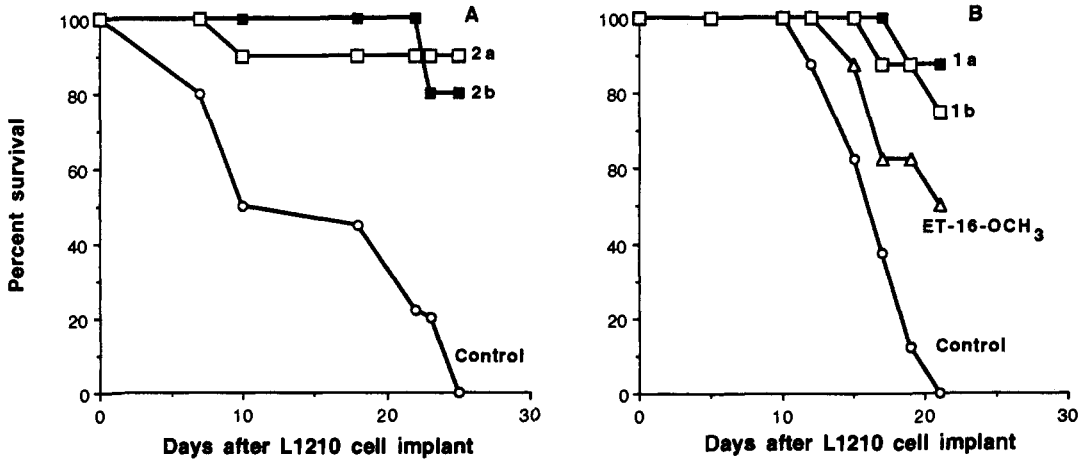


Figure 5. Effects of compounds **2a** and **2b** (A) and of compounds **1a**, **1b**, and *rac*-ET-16-OMe (B) on the survival of CD1 mice injected with L1210 tumors. Each group contained 10 (A) or 8 (B) animals. Drug treatment (5 mg/kg per day, 5 days per week, intramuscularly) was initiated 3 days after injection of L1210 cells. Animal deaths were used as the index of lethality.

$[\alpha]^{28}_D$ −5.19° (c 5.0, C_6H_6); $[\alpha]^{28}_D$ −2.98° (c 5.0, $CHCl_3$); IR (KBr) 3660, 2356, 1214, 1061, 1032 cm^{-1}; NMR ($CDCl_3$) δ 3.74 (d, 6H, J = 10.8 Hz), 3.3–3.5 (m, 5H), 2.78 (br s, 1H), 1.7–1.9 (m, 4H), 1.2–1.7 (m, 2H), 1.26 (br s, 26H), 0.88 (t, 3H, J = 6.7 Hz). Anal. Calcd for $C_{22}H_{47}O_5P$: C, 62.53; H, 11.21; P, 7.33. Found: C, 62.78; H, 11.15; P, 7.60.

Dimethyl 4-(Hexadecyloxy)-3(*R*)-hydroxybutanephosphonate. The enantiomer of **5** was prepared in 90% yield from the epoxide by the procedure described above; $[\alpha]^{25}_D$ +5.20° (c 5.0, C_6H_6).

Dimethyl 4-(Hexadecyloxy)-3(*S*)-methoxybutanephosphonate (6). Method A. To a mixture of 2.12 g (5.0 mmol) of hydroxy phosphonate **5** and 11 g of silica gel (previously heated at 150 °C for 2 h under high vacuum) was added an ether solution of diazomethane (20 molar equiv based on substrate) at 0 °C. After the mixture had stirred at 0 °C for 6 h, another 20 molar equiv of diazomethane solution was added, and the mixture was stirred for 24 h at 0 °C. The silica gel was removed from the reaction mixture by filtration and washed with ether. The product was purified by flash column chromatography on silica gel (elution with chloroform–methanol 50:1) to give 1.94 g (88%) of methoxy phosphonate **6** as a colorless oil.

Method B. To a solution of 2.12 g (5.0 mmol) of hydroxy phosphonate **5** and 2.05 g (10 mmol) of 2,6-di-*tert*-butyl-4-methylpyridine in 20 mL of dichloromethane was added 1.1 mL (9.7 mmol) of methyl triflate. The reaction mixture was heated at reflux for 16 h under nitrogen. The product was partitioned between 2 N hydrochloric acid and chloroform. The organic layer was washed with water and saturated aqueous sodium bicarbonate solution and dried over sodium sulfate. Removal of the solvents and purification by column chromatography gave 1.96 g (89%) of methoxy phosphonate **6**: R_f 0.61 (9:1 chloroform–methanol); $[\alpha]^{28}_D$ −6.71° (c 5.0, $CHCl_3$); IR (film) 2356, 1361, 1114, 1061, 1020 cm^{-1}; NMR ($CDCl_3$) δ 3.74 (d, 6H, J = 10.8 Hz), 3.40 (s, 3H), 3.3–3.5 (m, 5H), 1.7–1.9 (m, 4H), 1.2–1.7 (m, 2H), 1.26 (br s, 26H), 0.88 (t, 3H, J = 6.7 Hz). Anal. Calcd for $C_{23}H_{49}O_5P$: C, 63.27; H, 11.31; P, 7.09. Found: C, 62.97; H, 11.44; P, 6.99.

Dimethyl 4-(Hexadecyloxy)-3(*R*)-methoxybutanephosphonate. The enantiomer of **6** was prepared in 90% yield from the epoxide by the procedure described above; $[\alpha]^{22}_D$ +6.78° (c 5.0, $CHCl_3$).

2′-(Triethylammonio)ethyl 4-(Hexadecyloxy)-3(*S*)-methoxybutanephosphonate (1a). To a solution of 437 mg (0.1 mmol) of methoxy phosphonate **6** in 5 mL of methylene chloride was added 1.3 mL (2.7 mmol) of bromotrimethylsilane. After the mixture was allowed to stand for 2 h at room temperature, volatile materials were removed under vacuum. The residue was dissolved in THF–water (17 mL, 8:1 by volume), and the mixture was allowed to stand for 2 h at room temperature. The solvents were removed under vacuum, and the residue was dried by repeated azeotropic distillation with dry 2-propanol under vacuum. Lyophilization from benzene gave 408 mg (100%) of the corresponding phosphonic acid as a white solid; mp 66.0–68.0 °C. The completion of the ester hydrolysis reaction was confirmed by the loss of the doublet at δ 3.74 ppm in the ^1H NMR spectrum. A stirred solution of the dry phosphonic acid, dry choline tosylate (1.5 g, 5.4 mmol), and trichloroacetonitrile (1.5 mL, 15 mmol) in pyridine (20 mL) was heated for 48 h at 50 °C (oil bath temperature). After most of the pyridine was removed, a dark brown semisolid residue was obtained, which was dissolved in THF–water (10 mL, 9:1 by volume) and passed through a

column of Amberlite MB-3 (previously equilibrated with the same solvent mixture). Pure phosphonocholine **1a** was obtained by silica gel column chromatography (elution with chloroform–methanol–ammonium hydroxide–water, 65:35:3:2): yield, 690 mg (69%); R_f 0.40 (65:35:8) chloroform–methanol–water); $[\alpha]^{28}_D$ −2.00° (c 1.0, $CHCl_3$/MeOH 1:1); IR (KBr) 3355, 1351, 1099, 963, 898 cm^{-1}; NMR ($CDCl_3$) δ 4.32 (m, 2H), 3.85 (m 2H), 3.45 (s, 9H), 3.39 (s, 3H), 3.1–3.7 (m, 5H), 1.5–1.9 (m, 6H), 1.2 (br s, 26H), 0.88 (t, 3H, J = 6.7 Hz). Anal. Calcd for $C_{28}H_{56}O_5PN·2H_2O$: C, 58.95; H, 11.42; N, 2.64; P, 5.85. Found: C, 58.61; H, 11.46; N, 2.56; P, 6.00.

2′-(Triethylammonio)ethyl 4-(Hexadecyloxy)-3(*R*)-methoxybutanephosphonate (1b). The enantiomer of **1a** was prepared in 70% yield from the dimethyl phosphonate by the procedure described above; $[\alpha]^{28}_D$ + 1.90° (c 1.0, $CHCl_3$/MeOH, 1:1).

Synthesis of the Enantiomers of 2′-(Trimethylammonio)-ethyl 4-(Hexadecylthio)-3-methoxybutanephosphonate (2a,b) (Scheme 2). 1-(Hexadecylthio)-*sn*-glycerol (7a). This compound was prepared as described elsewhere.[5]

3-(Hexadecylthio)-*sn*-glycerol (7b). This compound was prepared as described elsewhere.[5]

Hexadecyl (*S*)-2-Oxiranylmethyl Thioether (8). A mixture of 3.33 g (10 mmol) of diol **7a**, 3.94 g (15 mmol) of triphenylphosphine, and 2.4 mL (15 mmol) of diethyl azodicarboxylate in 50 mL of benzene was refluxed for 24 h. After removal of the solvent, 50 mL of ether was added, and the precipitate of phosphine oxide was removed by filtration. The filtrate was concentrated under vacuum, and the residue was purified by flash chromatography (elution with 20:1 hexane/ethyl acetate) to give 2.71 g (86%) of the product as a white solid; mp 40–41 °C; R_f 0.81 (hexane/ethyl acetate, (9:1); $[\alpha]^{25}_D$ −5.25° (c 2.5, chloroform); IR (KBr) 2954, 2908, 2848, 1467, 1207, 1190, 1137, 1084, 1037, 955 cm^{-1}; NMR δ 3.21–3.09 (m, 1H), 2.91–2.55 (m, 6H), 1.71–1.52 (m, 2H), 1.26 (s, 26H), 0.88 (t, 3H, J = 6.8 Hz). Anal. Calcd for $C_{19}H_{38}SO$: C, 72.55; H, 12.18; S, 10.19. Found: C, 72.74; H, 12.37; S, 10.44.

Hexadecyl (*R*)-2-Oxiranylmethyl Thioether. This compound was prepared in 88% yield from diol **7b** by the procedure outlined above; $[\alpha]^{25}_D$ +5.12° (c 2.5, $CHCl_3$).

Dimethyl 4-(Hexadecylthio)-3(*S*)-hydroxybutanephosphonate (9). To a solution of 2.86 g (20 mmol) of dimethyl methanephosphonate in 15 mL of dry THF was added dropwise 8 mL (20 mmol) of *n*-butyllithium (a 2.5 M solution in hexane). After the reaction mixture was stirred for 30 min at −78 °C, 2.5 mL (20 mmol) of boron trifluoride etherate was added dropwise, followed by a solution of 1.58 g (5.0 mmol) of epoxide **8** in 50 mL of THF. The reaction mixture was stirred for 3 h at −78 °C, warmed to −20 °C, stirred for 1 h, and then quenched by the addition of saturated aqueous ammonium chloride solution. The mixture was concentrated under reduced pressure, and product **9** was worked up as described for compound **5**. Purification by flash chromatography (elution with chloroform–methanol, 25:1) gave 1.98 g (90%) of pure hydroxy phosphonate **9** as a white waxy solid after lyophilization from hexane: mp 38.5–40.5 °C; R_f 0.51 (chloroform–methanol, 9:1); $[\alpha]^{25}_D$ −2.09° (c 2.5, C_6H_6); IR (KBr) 3378, 2919, 2849, 1461, 1225, 1049, 896, 814, 755 cm^{-1}; NMR ($CDCl_3$) δ 3.74 (d, 6H, J = 10.8 Hz), 3.3–3.5 (m, 1H), 2.74–2.60 (m, 4H), 1.99–1.77 (m, 4H), 1.61–1.41 (m, 2H), 1.26 (s, 26H), 0.88 (t, 3H, J = 6.7 Hz). Anal. Calcd for $C_{22}H_{47}SO_4P$: C, 60.24; H, 10.80; S, 7.31; P, 7.06. Found: C, 60.18, H, 10.75; S, 7.25; P, 6.95.

Dimethyl 4-(Hexadecylthio)-3(*R*)-hydroxybutanephosphonate. This compound was prepared in 90% yield from the epoxide by the procedure described above; $[\alpha]^{25}_D$ +2.01° (c 2.5, C_6H_6).

Dimethyl 4-(Hexadecylthio)-3(*S*)-methoxybutanephosphonate (10). A mixture of 2.12 g (5.0 mmol) of hydroxy phosphonate **9** and 11 g of activated silica gel was allowed to react with diazomethane in ether as described under the preparation of compound **6** (method A). The product was purified by flash chromatography (elution with chloroform–methanol, 50:1) to give 1.94 g (88%) of pure hexadecylthio methoxy phosphonate **10** as a colorless oil: R_f 0.83 (chloroform–methanol, 9:1); $[\alpha]^{25}_D$ −15.3° (c 2.5, $CHCl_3$); IR (KBr) 2919, 2849, 1461, 1225, 1049, 896, 814, 755 cm^{-1}; NMR δ 3.75 (d, 6H, J = 1.7 Hz), 3.38 (s, 3H), 3.45–3.26 (m, 1H), 2.74 and 2.67 (AB q, 1H, J = 5.2, 13.3 Hz), 2.60–2.48 (m, 3H), 1.99–1.77 (m, 4H), 1.61–1.41 (m, 2H), 1.26 (s, 26H), 0.88 (t, 3H, J = 6.7 Hz). Anal. Calcd for $C_{23}H_{49}SO_4P$: C, 61.03; H, 10.91; S, 7.08; P, 6.84. Found: C, 60.83; H, 10.84; S, 7.06; P, 6.80.

Dimethyl 4-(Hexadecylthio)-3(*R*)-methoxybutanephosphonate. This compound was prepared in 91% yield from the epoxide by the procedure described above; $[\alpha]^{25}_D$ +15.5° (c 2.5, $CHCl_3$).

2′-(Trimethylammonio)ethyl 4-(Hexadecylthio)-3(*S*)-methoxybutanephosphonate (2a). Methoxy phosphonate **10** (453 mg, 0.1 mmol) was subjected to hydrolysis by addition of bromotrimethylsilane followed by THF–water (8:1), as described for compound **6**. Lyophilization from benzene gave the corresponding phosphonic acid as a white solid, which was coupled to choline tosylate as described above. After the crude product was passed through a column of Amberlite MB-3 (elution with THF–water, 9:1), pure (hexadecylthio)methoxyphosphonocholine **2a** was obtained by silica gel column chromatography (elution with chloroform–methanol–water, 65:25:4): yield, 351 mg (69%); R_f 0.40 (65:35:8 chloroform–methanol–water); $[\alpha]^{25}_D$ −6.99° (c 1.0, $CHCl_3$/MeOH 1:1); IR (KBr) 3413, 2919, 2848, 1643, 1461, 1226, 1085, 1049, 963, 898 cm^{-1}; NMR ($CDCl_3$) δ 4.32 (m, 2H), 3.85 (m, 2H), (s, 9H), 3.39 (s, 3H), 3.1–3.7 (m, 1H), 2.7–2.4 (m, 4H), 1.5–1.9 (m, 6H), 1.2 (br s, 26H), 0.88 (t, 3H, J = 6.7 Hz). Anal. Calcd for $C_{28}H_{56}SO_4PN·3.5H_2O$: C, 54.53; H, 11.01; S, 5.60; N, 2.45; P, 5.40. Found: C, 54.11; H, 10.91; S, 5.33; N, 2.51; P, 5.58.

2′-(Trimethylammonio)ethyl 4-(Hexadecylthio)-3(*R*)-methoxybutanephosphonate (2b). This compound was prepared in 70% yield from the dimethyl phosphonate by the procedure outlined above; $[\alpha]^{25}_D$ +6.67° (c 1.0, $CHCl_3$/MeOH, 1:1).

Drugs. ADR was obtained from Adria Laboratories (Dublin, OH). *rac*-ET-16-OMe, *rac*-ET-18-OMe, and hexadecylphosphocholine were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Line Studies. WEHI-3B, L1210, and P388 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin G (100 units/mL), streptomycin (0.1 mg/mL), amphotericin B (0.25 μg/mL), and mercaptoethanol (0.1 μM). P388 and P388-ADR cells were obtained from American Type Culture Collection (Rockville, MD). P388-ADR cells were grown in the presence of ADR (0.1 μg/mL); vincristine at 10 μM did not kill the multidrug-resistant P388-ADR cells, but did kill the ADR-sensitive P388 cells. Cells were passaged frequently, using dilutions of 1:4 to 1:40. Cell viability was counted using 1×10^4 cells per well in 96-well tissue culture dishes. Drugs were added to the cells, and the number of viable cells was counted using the trypan blue dye exclusion assay.[6] Stock solutions of the drugs (100 mM) were prepared in ethanol, and subsequent dilutions were made in the culture medium. Clonogenic assays were carried out in medium containing 0.2% methyl cellulose.[1] A range of drug concentrations or buffer alone were added to the cells, and colonies were counted after 5 days.

In Vivo Assays. WEHI-3B or P388 cells (1×10^6) were implanted subcutaneously in the back of BALB/C mice. Drug treatment commenced 3 days after tumor implant. The animals received 5 mg/kg of each drug per day (5 days per week) intramuscularly. Tumor volumes were estimated by using a caliper. In the studies of median survival time of CD1 mice implanted with L1210 murine lymphoid leukemic cells (Figure 5), the effects of **2a** and **2b** compared with controls (no drug) were monitored using 10 animals in each group, whereas the effects of **1a**, **1b**, and ET-16-OMe were studied using 8 animals in each group.

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