Synthesis and Use of Novel Ether Phospholipid Enantiomers To Probe the Molecular Basis of the Antitumor Effects of Alkyllysophospholipids: Correlation of Differential Activation of c-Jun NH_2-Terminal Protein Kinase with Antiproliferative Effects in Neuronal Tumor Cells

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Received June 4, 2003

The enantiomers of a novel unsaturated phosphonocholine antitumor ether lipid were synthesized and found to have differential antiproliferative effects against epithelial cancer cell lines. The basis of the enantioselective effects on the cells was investigated in SK-N-MC and SK-N-SH neuroblastoma tumor cells. Our results indicate that the enantioselective antiproliferative potency arises primarily from the activation of the JNK signaling pathway by the ether lipids.

Introduction

Antitumor ether lipids (AELs) represent a class of potential agents for the clinical treatment of cancer.[1−3] The prototypes of the AELs are 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycerophosphocholine (*rac*-ET-18-OCH_3) and its C16 analogue, ET-16-OCH_3 (**1**, Figure 1), which inhibit the growth of a broad panel of tumor cell lines.[4,5] A current interest is the preparation of hydrolytically stable analogues of AELs in order to enhance metabolic stability. Thus, we previously prepared the glycosidase-resistant *C*-glycosides **2** and **3** (X = CH_2) and compared their activities with the corresponding *O*-glycosides (X = O).[6,7] Unlike currently available chemotherapeutic compounds, these unnatural phospholipids do not interact with cellular DNA and therefore are not mutagenic.[1,8] Studies to unravel the mechanism of action of AELs are bedeviled by the unusually large number of cellular events reported to be affected by these compounds. These include induction of apoptosis via the generation of reactive oxygen species or JNK activation, inhibition of phosphatidylcholine synthesis, nutrient starvation, and inhibition of cellular signaling molecules including mitogen activated protein kinase (MAPK) and phospholipases.[1,9−12]

The development of closely related AEL compounds with differential antiproliferative and cytotoxic effects on cells could shed light on which cellular events are critical in the mechanism of action of AELs. Several previous studies of the role of chirality in AEL potency against tumor cells in vitro have demonstrated virtually no difference between the enantiomeric forms. These studies include the enantiomers of ET-16-OCH_3,[13] saturated phosphonocholine analogues of ET-16-OCH_3,[14] 2′-(trimethylammonio)ethyl 3-(hexadecyloxy)-2-(methoxymethyl)propanephosphate (oxo-BM41.440),[15] and

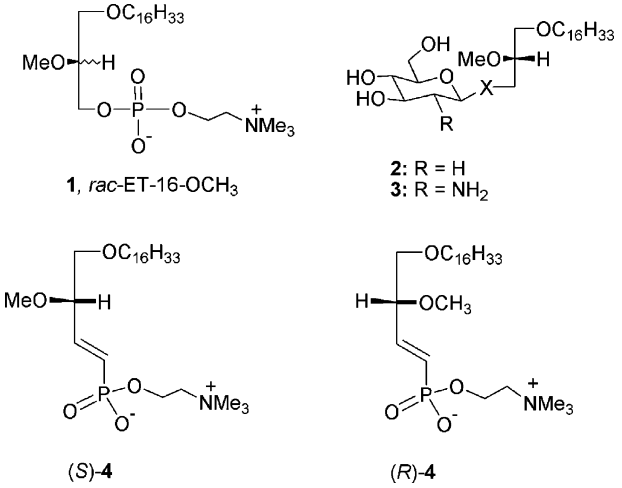

Figure 1.

2-[[hydroxy[[2-[(octadecyloxy)methyl]tetrahydrofuran-2-yl]methoxy]phosphinyl]oxy]-*N*,*N*,*N*-trimethylethylaminium hydroxide salt (SRI 62.834).[16−18] We describe herein the synthesis of 2′-(trimethylammonio)ethyl 4-(hexadecyloxy)-3-methoxy-1-butenephosphonate ((*R*)- and (*S*)-**4**); these are enantiomeric hydrolytically stable isosteres of the phosphate ester ET-16-OCH_3 (**1**). Surprisingly, we found that this enantiomeric pair has markedly different antiproliferative and cytotoxic effects in some epithelial cancer cell lines. The enantiomers of the unsaturated phosphonocholines **4** are the first example of AELs that exhibit enantioselective action on the proliferation of cancer cell lines in vitro. We demonstrate that this pair of compounds offers a novel means for relating the postulated cellular effects of AELs to the mechanism of inhibition of cell growth.

Results and Discussion

Chemistry. (*S*)-**4** was synthesized as shown in Scheme 1. Swern oxidation of 1-*O*-hexadecyl-2-*O*-methyl-*sn*-glycerol ((*S*)-**5**), prepared in 91% ee as described previ-

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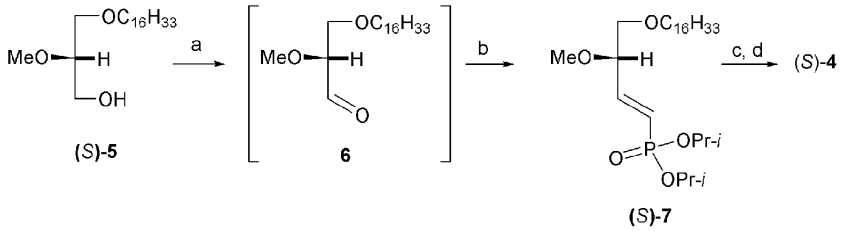
10.1021/jm0302748 CCC: $27.50
Published on Web 04/03/2004

Scheme 1[a]



[a] (a) (i) $(COCl)_2$, DMSO, 1 h, −78 °C, (ii) Et_3N; (b) $NaCH[P(O)(OPr\text{-}i)_2]_2$, THF, 0 °C; (c) (i) TMSBr, CH_2Cl_2, 2 h, (ii) THF/H_2O (8:1), (iii) choline tosylate, Cl_3CCN, py, 48 h, 50 °C.

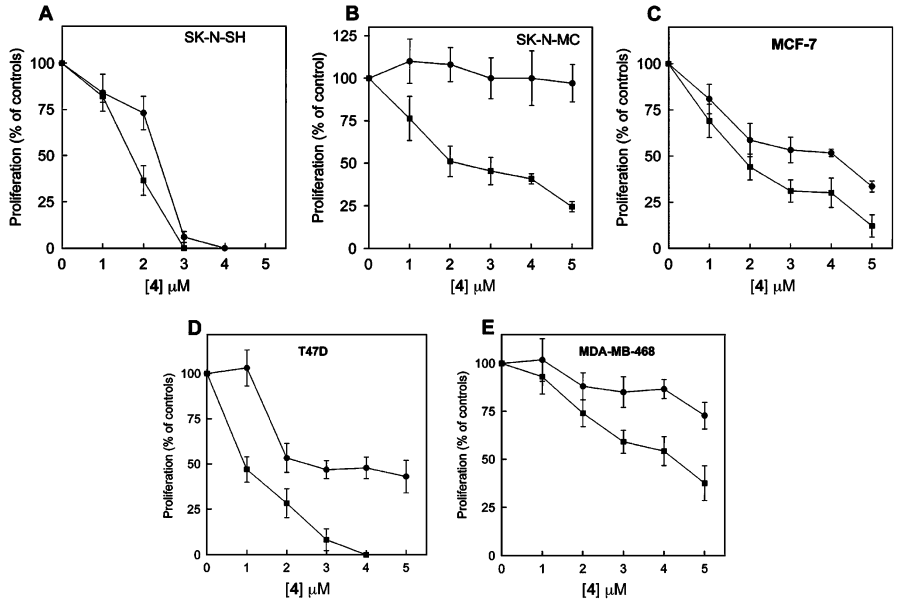


Figure 2. Effect of (*R*)- or (*S*)-**4** on the proliferation of epithelial cancer cell lines. Proliferating cells were incubated in 24-well plates with medium containing 0−5 μM of (*R*)- (■) or (*S*)-**4** (●). Cells from representative wells were counted on day 0 prior to the addition of **4**. After 48 h, the cell numbers were determined in each well and the increase in numbers over day 0 for each concentration was expressed as a percentage of that in control wells with no drug. The results are the means and standard deviations of two different experiments with quadruplicate wells per experiment.

ously,[19] followed by reaction of the resulting aldehyde **6** with sodium tetraisopropyl methylenediphosphonate, afforded phosphonate ester **7** in 88% overall yield. Hydrolysis of the isopropyl ester groups of **7** and coupling of the resultant phosphonic acid to choline tosylate provided the target (*S*)-**4** (61% for the two steps). (*R*)-**4** was synthesized in an analogous manner to (*S*)-**4** starting from (*R*)-**5**.

Biological Studies. To assess the antiproliferative effects of the enantiomers, exponentially growing epithelial cancer cells, two neuroblastoma cell lines (SK-N-SH and SK-N-MC), and three breast cancer cell lines (MCF-7, MDA-MB-468, and T47D) were incubated with 0−5 μM of (*R*)- or (*S*)-**4** for 48 h, and the increase in cell numbers relative to controls was measured. The results are displayed in Figure 2. With the exception of SK-N-SH cells where (*R*)- and (*S*)-**4** had the same activity, in all the cell lines (*S*)-**4** was less cytostatic than (*R*)-**4**. The ability of the adhering cells to exclude trypan blue dye was determined after incubation with 5 μM of the phosphonocholine enantiomers. Both enantiomers completely killed the SK-N-SH cells, and there were no attached cells at the end of the incubation period. (*S*)-**4** had no effect on the viability of the other cell lines. Even though (*R*)-**4** inhibited the proliferation of all the cells, the viability of the remaining SK-N-MC, T47D, and MDA-MB-468 cells was between 92−94%, which was similar to that of non-drug-treated cells. However, the viability of MCF-7 cells was only 61%. Thus, (*R*)-**4** was more cytotoxic to MCF-7 cells than (*S*)-**4**. These results represent the first report of enantioselectivity on the antiproliferative and cytotoxic effects of AELs. The basis of the enantioselective effect is unknown, but is likely to be related to the introduction of the double bond since the saturated phosphonocholines did not exhibit an enantioselective effect.[14]

Perturbations of c-Jun-NH_2-terminal kinase (JNK),[20] protein kinase B (PKB),[11] and the mitogen-activated protein kinase (MAPK)[21] pathways by alkylysophospholipids have been implicated in the mechanism of action of antitumor ether lipids. The differential effects of the enantiomeric AELs provide an opportunity to examine the roles of these pathways in cell proliferation. The neuroblastoma SK-N-MC and SK-N-SH cells were used for these studies because (*R*)- and (*S*)-**4** had significantly different effects on the former, but similar effects on the latter cells. To examine the effects of the compounds on signaling molecules, we used quiescent cells in order to obtain a synchronized response that would be more readily detected.[21] The concentration and incubation time with (*R*)-**4** that were cytostatic for quiescent SK-N-MC cells were established (15 μM for 4 h) by previously described procedures[21] and used for all experiments.

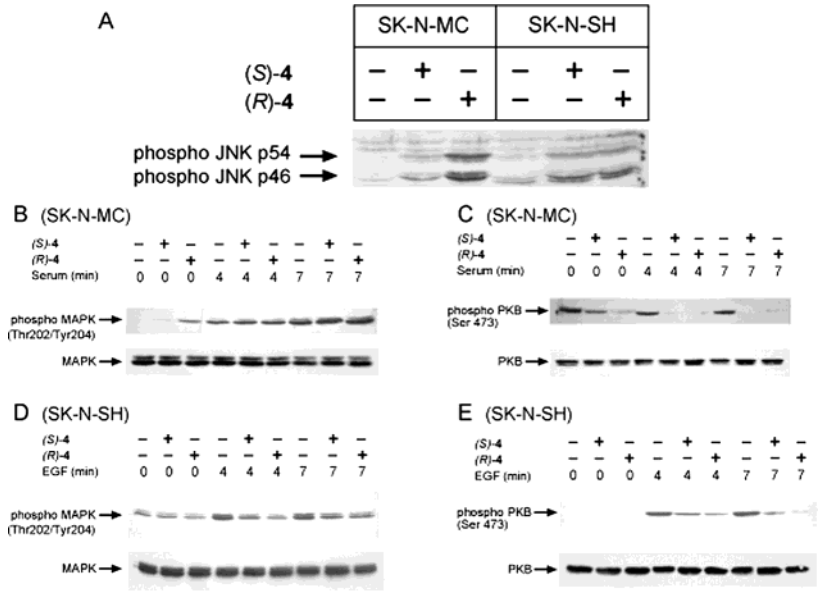


Figure 3. Effects of (*R*)- and (*S*)-**4** on JNK, MAPK, and PKB phosphorylation in quiescent SK-N-MC and SK-N-SH cells. (A) Quiescent cells were incubated with or without (*R*)- and (*S*)-**4** (15 μM) for 4 h. Cell lysates were prepared and proteins were separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes or Western blot analysis with anti-phospho-specific JNK (Thr 183/Tyr 185). (B−E) Quiescent SK-N-MC (B and C) and SK-N-SH (D and E) cells were incubated with or without (*R*)- and (*S*)-**4** (15 μM) for 4 h. The medium was replaced with one without the drugs, the cells were stimulated with 10% FBS-supplemented DMEM (SK-N-MC) or EGF (SK-N-SH) and washed, and cell lysates were prepared as described in Methods. Immunoblotting was performed with anti-phospho-specific MAPK (Thr 202/Tyr 204) antibody (B, D) and anti-phospho-PKB (Ser 473) antibody (C, E). The results are from a single experiment that is representative of results obtained with three different cell preparations for each cell line.

The results of the effects of (*R*)-and (*S*)-**4** on the activation of the JNK, MAPK, and PKB pathways in SK-N-MC cells are shown in Figures 3A, 3B, and 3C. In these cells (*R*)-**4** inhibited proliferation but (*S*)-**4** did not. Activation of JNK was 5−8-fold higher in quiescent SK-N-MC cells incubated with (*R*)-**4** than with (*S*)-**4** (Figure 3A). The effects of the compounds on JNK activation therefore correlated with that of the enantiomers on the proliferation of SK-N-MC cells. SK-N-MC cells do not have epidermal growth factor (EGF) receptors[22] and showed little response to insulin in preliminary studies. Therefore, the cells were stimulated with 10% fetal bovine serum (FBS)-supplemented medium to activate the MAPK and PKB pathways. In SK-N-MC cells stimulated with 10% FBS-supplemented medium, both enantiomers had no effect on the activation of MAPK (Figure 3B) but they both similarly inhibited PKB activation by 10−12-fold relative to the PKB activity in control cells not treated with any drug. Since both enantiomers effectively inhibited PKB but displayed significant differences in the antiproliferative effects which correlated with their effect on JNK activation, we conclude that the consequences of AEL-induced JNK activation supersede the contribution of PKB inhibition to the overall cellular outcome.

The results of similar studies with SK-N-SH cells are shown in Figure 3A, 3D, and 3E. As with SK-N-MC cells, the AEL-induced activation of JNK is the dominant effect that is correlated with the activity of the enantiomers in SK-N-SH cells. (*R*)- and (*S*)-**4** activated JNK (Figure 3A) similarly (2.5-fold activation) and inhibited MAPK activation (Figure 3D) to the same degree (2-fold inhibition), but they differed significantly in the degree of PKB inhibition (Figure 3E). (*S*)-**4** inhibited PKB activation by 4-fold whereas (*R*)-**4** inhibited the activation by 12-fold. This differential effect on PKB inhibition did not result in significant differences in the antiproliferative or cytotoxic effects of the enantiomers against SK-N-SH cells. As inhibition studies with SK-N-MC showed that MAPK inhibition is not obligatory for growth inhibition (Figure 3B), the effect of the enantiomers on the activation of the signaling molecules in SK-N-SH cells is consistent with the view that activation of JNK by AEL is the predominant event that dictates the cellular end-point.

The relevance of JNK activation in the cytotoxic action of AELs is supported by the following observations: (a) inhibitory concentrations of ET-18-OCH_3 activate JNK in quiescent MCF-7 cells (Figure 1, Supporting Information) and (b) addition of a JNK inhibitor, SP600125,[23] to proliferating MCF-7 cells prior to incubation with ET-18-OCH_3 or (*R*)-**4** prevents cell death (Figure 2, Supporting Information).

In conclusion, we have synthesized novel unsaturated phosphonocholines with enantioselective effects on cell proliferation and viability. We used (*R*)- and (*S*)-**4** to demonstrate a correlation between the activation of JNK and the differential cytostatic/cytotoxic effects of the compounds. The availability of these enantiomeric compounds should permit further study of the relevance of the numerous cellular events purported to play a role in the cytostatic and cytotoxic action of AELs with the ultimate goal of identification of their principal molecular targets.

Experimental Section

Chemistry. Diisopropyl 4-(Hexadecyloxy)-3-(*S*)-methoxy-1-(*E*)-butenephosphonate (7). Dimethyl sulfoxide (1.85 mL, 21.4 mmol) was added to a solution of oxalyl chloride (1.0 mL, 11.5 mmol) in 150 mL of methylene chloride at −78 °C. After 10 min, 1-*O*-hexadecyl-2-*O*-methyl-*sn*-glycerol ((*S*)-**5**, 3.31 g, 10.0 mmol) in 20 mL of methylene chloride was added, and

the reaction mixture was stirred for 1 h at −78 °C, followed by the addition of triethylamine (7.0 mL, 50 mmol). After 1 h at −78 °C, the mixture was warmed to room temperature, diluted with Et_2O, and washed with water, 10% aqueous sodium bisulfate solution, and brine solution. The organic phase was dried (Na_2SO_4) and concentrated to give crude methoxyglyceraldehyde **6**. Tetraisopropyl methylenediphosphonate (3.44 g, 10 mmol) was added to a suspension of sodium hydride (0.3 g, 10 mmol, 85% in white oil, washed with dry hexane twice) in 50 mL of dry THF at 0 °C. After cessation of H_2 evolution, crude aldehyde **6** was added in 10 mL of THF and the mixture was stirred overnight at 0 °C. The mixture was concentrated under reduced pressure, the residue was dissolved with Et_2O and then washed with water and brine solution. The organic phase was dried (Na_2SO_4) and concentrated. The residue was purified by column chromatography on silica gel (elution with chloroform−methanol 50:1) to give 4.31 g of diisopropyl 4-(hexadecyloxy)-3-(*S*)-methoxy-1-(*E*)-butenephosphonate (**7**) (88%) as a pale yellow oil; $[\alpha]^{25}_D$ −3.14° (*c* 50.8, $CHCl_3$). Anal. Calcd for $C_{27}H_{55}O_5P$: C, 66.09; H, 11.30; P, 6.31. Found: C, 66.21; H, 11.22; P, 6.26.

2′-(Trimethylammonio)ethyl 4-(Hexadecyloxy)-(3)-(*S*)-3-methoxy-1-(*E*)-butenephosphonate ((*S*)-4). To a solution of butenephosphonate ester **7** (246 mg, 0.50 mmol) in 10 mL of methylene chloride was added bromotrimethylsilane (1.3 mL, 2.7 mmol). After the reaction mixture had been stirred for 2 h at room temperature, the volatile materials were removed in vacuo, the residue was dissolved in THF-water (10 mL, 8:1 by volume), and the mixture was allowed to stand for 2 h at room temperature to complete the hydrolysis of the isopropyl ester groups. The solvents were removed with the aid of dry 2-propanol, followed by lyophilization from benzene, to give the corresponding phosphonic acid. A solution of the phosphonic acid, choline tosylate (0.30 g, 0.90 mmol), and trichloroacetonitrile (0.30 mL, 3.0 mmol) in 20 mL of pyridine was heated for 48 h at 50 °C. After most of pyridine was removed under reduced pressure, a dark brown semisolid residue was obtained. The residue was dissolved in THF−water (9:1 by volume) and passed through a column of Amberlite MB-3, which was previously equilibrated with THF-water (9:1). Pure phosphonocholine (*S*)-**4** was obtained after chromatography on silica gel column two times, eluting with chloroform−methanol−water (65:35:4); 150 mg (61% yield); $[\alpha]^{25}_D$ −2.84° (*c* 7.6, $CHCl_3$−CH_3OH, 1:1); Anal. Calcd for $C_{26}H_{54}O_5NP\ 2H_2O$: C, 59.18; H, 11.08; N, 2.65; P, 5.87. Found: C, 59.20; H, 11.11; N, 2.44; P, 5.66.

(*R*)-**4** was synthesized from 3-*O*-hexadecyl-2-*O*-methyl-*sn*-glycerol ((*R*)-**5**)[19] in 59% yield by an analogous procedure to that described above; $[\alpha]^{25}_D$ +2.83° (*c* 7.9, $CHCl_3$−CH_3OH, 1:1; Anal. Calcd for $C_{26}H_{54}O_5NP\ 3H_2O$: C, 57.22; H, 11.08; N, 2.65; P, 5.87. Found C, 57.86; H, 9.81; N, 2.86; P, 4.94.

Acknowledgment. This study was supported by a grant from the Canadian Institute of Health Research and a CUNY Research Award to R.B.

Supporting Information Available: Spectral analysis, biological procedures, and additional biological results showing the activation of JNK by ET-18-OCH_3 and prevention of cell death by a JNK inhibitor. This material is available free of charge via the Internet at http://pubs.acs.org.

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JM0302748