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Synthesis and antitumor properties of a plasmalogen methyl ether analogue

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Abstract—Incorporation of a *cis*-*O*-vinyl linkage into the *sn*-1 position of glycerol gave a new antitumor ether lipid analogue of ET-18–OCH_3 (**2**). We report here the synthesis of plasmalogen *O*-methyl ether **4** and its in vitro activity on epithelial cancer cells. The activity of **4** with respect to inhibition of the growth of several human breast cancer cells is slightly less than that of **2**. Surprisingly, incubation of SKBr3 and BT-20 (human breast cancer cells) with varying concentrations of **4** resulted in a marked stimulation of cell growth. The induction of cellular proliferation was not transduced by the Edg-2, -4, or -7 subfamilies of G-protein-coupled receptors.

1. Introduction

Plasmalogens (**1**) are naturally occurring[1] phospholipids with a *O*-*cis*-vinyl ether linkage at carbons 1 and 2 of the hydrocarbon chain attached to the *sn*-1 position of the glycerol backbone, a long-chain ester at the *sn*-2 position, and a phosphocholine or phosphoethanolamine group at the *sn*-3 position. The distinctive functions of plasmalogens[2] is apparently conferred by the unsaturated nature of their enol ether linkage. The susceptibility of this linkage to oxidation may be responsible for the protection provided by plasmalogens to endothelial cells and plasma lipoproteins against attack by reactive oxygen species.[3] The synthetic anticancer agent ET-18–OCH_3 (edelfosine, *rac*-**2**)[4] is structurally very similar to the natural product platelet-activating factor (PAF, 1-*O*-alkyl-2-*O*-acetyl-*sn*-glycero-3-phosphocholine, **3**).[5] Lipids **2** and **3** bear a saturated hydrocarbon chain in an ether linkage to the *sn*-1 position of glycerol. In PAF (**3**), an acetate group is linked to the *sn*-2 position of the glycerol backbone, whereas in antitumor ether lipid **2** an *O*-methyl group is linked to the *sn*-2 position. A great deal of effort has been directed to the synthesis and biological investigation of analogues of **2**.[6] We report here the synthesis and in vitro biological activity of a new analogue of **2**, the 2-*O*-methyl ether analogue of plasmalogen **4**.[7] Compounds **2** and **4** are both diether phospholipids; **4** differs from the prototypical antitumor ether lipid **2** by having a labile *O*-*cis*-1′-alkenyl linkage at the *sn*-1 position, and is also chiral, whereas **2** is racemic. We found that while both **2** and **4** were effective growth inhibitors of several human breast cancer cell lines, the new analogue **4** was able to markedly stimulate the growth of two other breast cancer cell lines (SKBr3 and BT-20). We also determined that the mitogenic response of these cells to **4** is independent of the endothelial differentiation gene (Edg) receptors Edg-2, -4, and -7.

2. Results and discussion

2.1. Synthesis of plasmalogen *O*-methyl ether analogue 4

The synthesis started with commercially available (*S*)-isopropylidene-glycerol (**5**), which was converted to 3-*O*-*p*-methoxybenzyl (PMB)-*sn*-glycerol (**6**) in quantitative overall yield as described previously.[8] PMB diol **6** was tritylated regioselectively at the primary position (trityl chloride, pyridine, DMAP, CH_2Cl_2) to give 1-*O*-trityl-3-*O*-PMB-*sn*-glycerol (**7**) as described previously.[8] *O*-Methylation of **7** with KH and MeI, followed by detritylation (*p*-TsOH, MeOH), afforded 2-*O*-methyl-3-*O*-PMB-*sn*-glycerol (**8**) in 90% overall yield. The *O*-vinyl ether moiety was introduced at the *sn*-1 position via the reaction sequence shown in Scheme 1. First, *O*-alkynyl ether glycerol derivative **9** was prepared in 70% overall yield from 2-*O*-methyl-3-*O*-PMB-*sn*-glycerol (**8**) by reaction with KH and trichloroethylene in THF, followed by *n*-BuLi in hexane to give an *O*-alkynyllithium intermediate, which was alkylated with 1-iodohexadecane in HMPA. This method for introducing an

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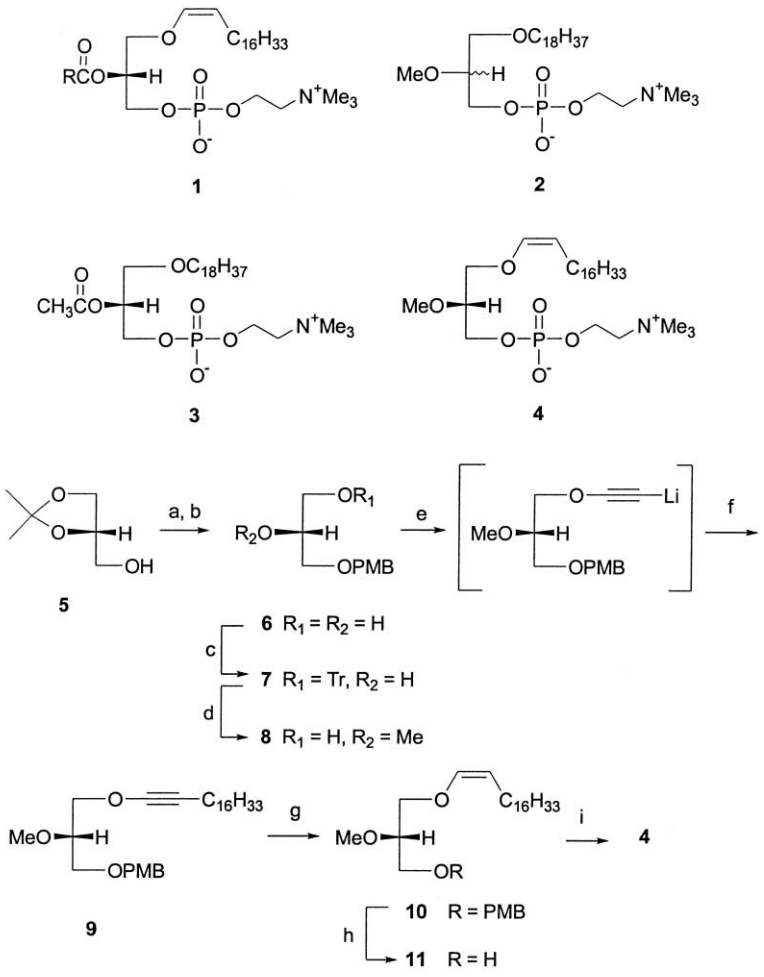

Scheme 1. Synthesis of plasmalogen methyl ether analogue **4**. *Reagents and conditions*: (a). KH, PMBCl, THF; (b). *p*-TsOH, MeOH (100% in two steps); (c). TrCl, Py, DMAP, CH_2Cl_2 (100%); (d). (i) KH, MeI, THF, (ii) *p*-TsOH, MeOH (90%); (e). (i) KH, CHCl=CCl_2, −42°C to rt, 2 h; (ii) *n*-BuLi, −78 to −42°C; (f). $C_{16}H_{33}I$, HMPA, −42°C to rt, 2 h (69%); (g). H_2/Lindlar catalyst, quinoline, hexane/EtOAc 1:1, 2 h (100%); (h). Na/NH_3, −78°C to rt (90%); (i). (i) 2-chloro-2-oxo-1,3,2-dioxaphospholane, Py, C_6H_6, 4°C, overnight; (ii) NMe_3, MeCN/benzene 3:1, 65°C, 48 h (68%).

O-alkynyl linkage, which was originally found to be successful for the preparation of short-chain acetylenic ethers,[9] was applied to long-chain alkynyl ethers in our total synthesis of a plasmalogen bearing a palmitoyl group at the *sn*-2 position.[8] The next step was the partial hydrogenation of the *O*-octadecynyl group to a *cis*-*O*-octadecenyl group. The product was obtained in 100% yield by using Lindlar catalyst, which was poisoned with quinoline, in hexane/EtOAc 1:1. Although an inseparable mixture of *O*-alkenyl isomers was obtained, the *Z*/*E* stereoselectivity was high. A *Z*/*E* ratio of 30:1 was found in alkenyl glycerol derivative **10**, as evidenced by ^{1}H NMR integration of the vinyl proton at δ 5.9 ppm (*Z*) and 6.2 ppm (*E*). Since the *O*-alkenyl ether linkage is highly acid labile, basic conditions are needed for the conversion of **10** to the target compound, **4**. Deprotection of the PMB group, without affecting the stereochemistry of the *O*-vinyl ether moiety, was accomplished by using sodium metal in liquid ammonia, giving alcohol **11** in 90% yield. The phosphocholine moiety was introduced in 68% yield by reaction of alcohol **11** with 2-chloro-2-oxo-1,3,2-dioxaphospholane in the presence of pyridine in benzene at 4°C, followed by ring opening with NMe_3 in MeCN/benzene (3:1) in a pressure tube at 65°C for 48 h.

2.2. Biological

The effect of plasmalogen analogue **4** on the proliferation of epithelial cancer cell lines derived from breast tissue was investigated. The plasmalogen analogue proved to be cytotoxic against MCF-7, MDA-MB-231, and SKBr3 cells, and also significantly inhibited the proliferation of MDA-MB-468 and T47D cells (Figs. 1 and 2). Surprisingly, **4** stimulated the proliferation of BT-20 cells at all concentrations examined (Fig. 2). The highest stimulation was a 5- to 6-fold increase at a drug concentration of 7.5 μM (Fig. 2). A significant stimulation of SKBr3 cell proliferation was also observed at lower concentrations of **4** (2.5–7.5 μM). Higher concentrations of **4** led to a decrease in SKBr3 cell proliferation and cell death (IC_{50} 26 μM).

A comparison of the effects of **4** and the parent compound ET-18–OCH_3 (**2**) revealed that **4** was less potent than **2** with respect to inhibitory and cytotoxic properties (Fig. 1). Significant differences between **2** and **4** were observed in their growth-stimulating properties (Fig. 2). At low concentrations, plasmalogen analogue **4** stimulated SKBr3 cell growth, whereas ET-18–OCH_3 inhibited it. Low concentrations of ET-18–OCH_3 (<7.5 μM) stimulated BT-20

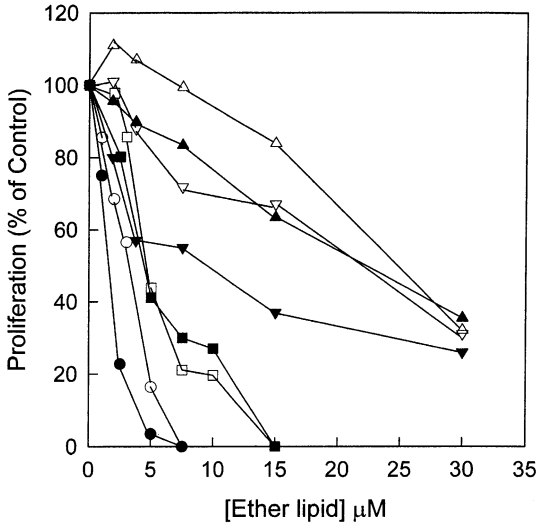

Figure 1. Effects of **2** and **4** on the proliferation of MCF-7 (○), MDA-MB-231 (□), MDA-MB-468 (△), and T47D (▽) breast cancer cell lines. Proliferating cells growing in 24-well plates were incubated with medium containing compound **2** (closed symbols) or **4** (open symbols). Cells in representative wells were counted on 0 day prior to the addition of the compound. Forty-eight hours after the addition of the compound the cell numbers were determined, and the increase over day 0 for each concentration was expressed as a percentage of that in control wells that did not receive the compound. The results are the means of two different experiments with quadruplicate wells/experiment. The standard deviations from the means are less than 14%.

cell proliferation by 2.5 fold, whereas the same concentration of plasmalogen analogue **4** stimulated BT-20 cell growth by 5- to 6-fold. Importantly, higher ET-18–OCH_3 concentrations were cytotoxic to BT-20 cells, whereas a 3-fold increase in stimulation was still observed with 30 μM of plasmalogen analogue **4**.

The inhibitory effect of ET-18–OCH_3 and related antitumor

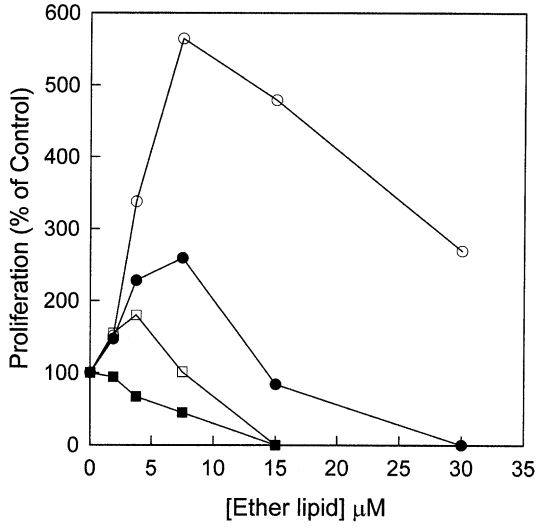

Figure 2. Effects of **2** or **4** on the proliferation of SKBr3 (□) and BT-20 (○) breast cancer cell lines. Experiments were carried out as in Fig. 1. Cells incubated with **2** are represented by the closed symbols; cells incubated with **4** are represented by the open symbols. The results are the means of two different experiments with quadruplicate wells/experiment. The standard deviations from the means are less than 14%.

ether lipids on cell proliferation appears to be related to the perturbation of cell signaling pathways involved in cell proliferation[4c] while the cytotoxic effects may be related to induction of apoptosis by these compounds.[10] The similarities in structure and cytotoxicity profiles of **2** and **4** suggest that similar mechanisms may be responsible for their growth inhibitory effects. The molecular basis for the very potent cell-specific growth-stimulatory effects of plasmalogen analogue **4** is not yet known. PAF (**3**), lyso-PAF, and structurally related phospholipids are known to induce mitogenic activity via stimulation of growth factors.[11,12] It is possible that *O*-alkenyl-glycerophosphate analogs that interact with phospholipid growth factor receptors[13] or α-hydroxyaldehydes, putative growth regulating second messengers[14] that could be potentially generated from **4**, may mediate the growth stimulatory effects.

To investigate whether growth stimulation by phospholipid **4** may be mediated via the subfamilies of G-protein-coupled growth factor receptors Edg-2, Edg-4, Edg-7, and/or psp24[12,13] we examined their mRNA expression in the different cell lines by the reverse transcriptase-polymerase chain reaction (RT-PCR). Fig. 3 shows that T47D, MCF-7, BT-20, and SKBr3 expressed only the Edg-4 receptor. MDA-MB-231 expressed Edg-4 and Edg-2 whereas MDA-MB-468 expressed Edg-4 and Edg-7 and a small amount of Edg-2 receptor mRNA (Fig. 3). Since the enhancement of cell proliferation on incubation with plasmalogen analogue **4** was observed in only BT-20 and SKBr3 cells, these results suggest that the mitogenic response does not depend on the expression of Edg-4 receptors. In addition, since the BT-20 and SKBr3 cell lines do not express Edg-2, Edg-7, or psp24 mRNA, it is unlikely that these receptors are involved in the mitogenic effect induced in these cell lines by **4**. Thus, it may be concluded that the mitogenic responses are mediated by pathways distinct from activation of Edg-2, -4, and -7 receptors.[15] The BT-20 cell line appears to be an excellent model for studies to elucidate the mechanism responsible for the unexpected mitogenic effects of the plasmalogen methyl ether analogue.

3. Experimental

3.1. General methods

For general procedures, see Ref. 16. 2-Chloro-2-oxo-1,3,2-dioxaphospholane was purchased from Fluka and stored at −80°C. Hexadecyl iodide was purchased from Lancaster Synthesis Ltd. 3-*O*-(4′-Methoxybenzyl)-*sn*-glycerol (**6**) and 1-*O*-triphenylmethyl-3-*O*-(4′-methoxybenzyl)-*sn*-glycerol (**7**) were synthesized as described previously.[8] The solvents were dried as follows: benzene (distilled from CaH_2); pyridine (dried over NaOH pellets); acetonitrile, methylene chloride, and chloroform (distilled from P_2O_5), HMPA (distilled from CaO). Elemental analyses were carried out by Desert Analytics (Tucson, AZ). ^1H and ^{13}C NMR spectra were recorded at 400 and 100 MHz on a Bruker spectrometer, respectively, and were referenced to the residual $CHCl_3$ at δ 7.24 (^1H) and δ 77.00 ppm (^{13}C).

3.1.1. 2-*O*-Methyl-3-*O*-PMB-*sn*-glycerol (8). To a solution of 1-*O*-trityl-3-*O*-PMB-*sn*-glycerol (**7**) (2.0 g, 4.40 mmol)

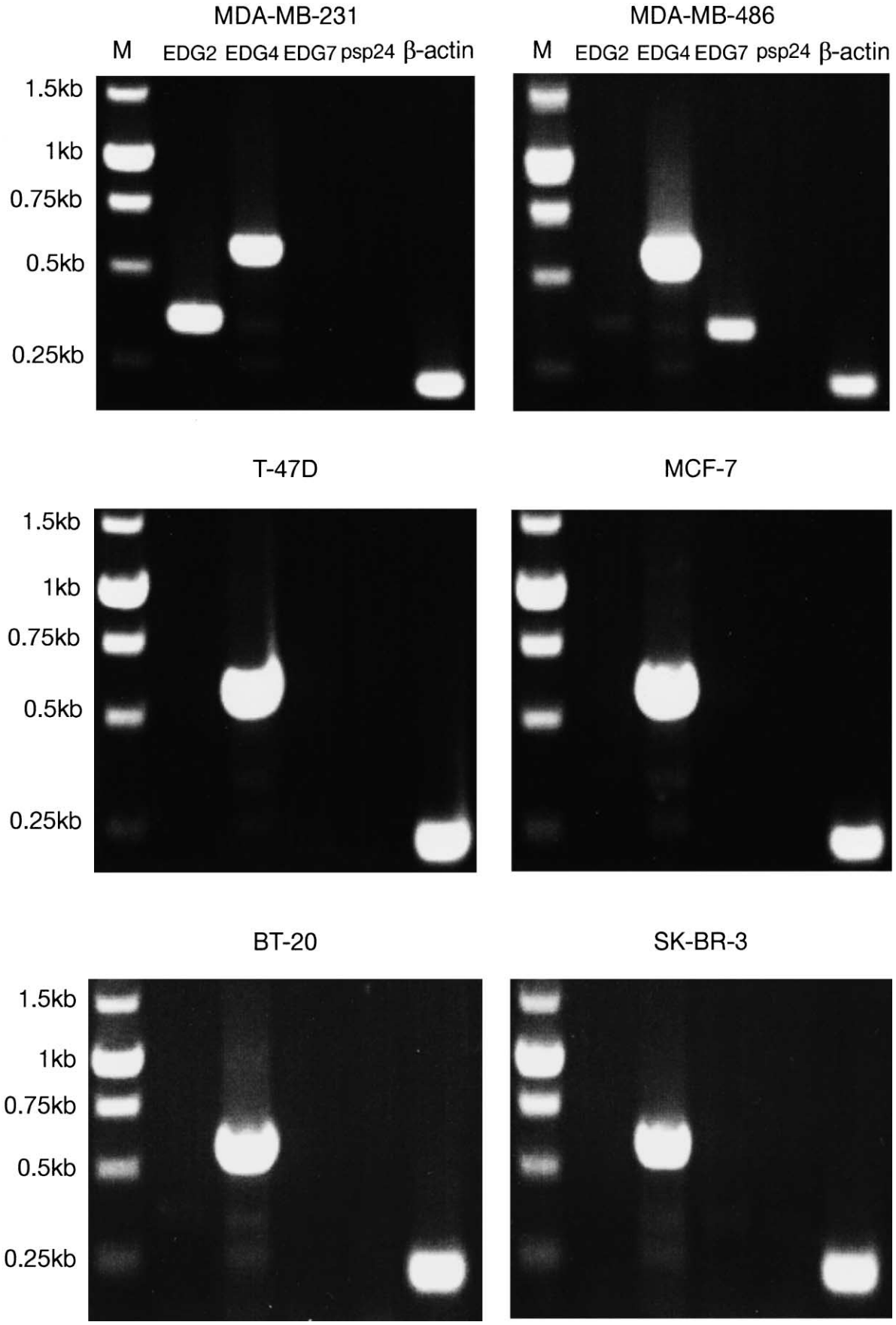

Figure 3. RT-PCR analysis of Edg-2, Edg-4, Edg-7, and psp24 receptor expression in cancer cell lines.

in 50 mL of THF was added KH (260 mg, 6.60 mmol). After the evolution of hydrogen ceased, MeI (1.25 g, 8.80 mmol) was added. After the solution was stirred at room temperature for 2 h, MeOH was added to destroy the excess KH. The solution was diluted with 100 mL of Et_2O and washed with saturated aqueous NH_4Cl solution and water. Evaporation of the solvents gave a residue that was dissolved in 50 mL of MeOH. The resulting solution was treated with *p*-TsOH (82 mg, 0.44 mmol) overnight at room temperature. The solution was neutralized with concentrated aqueous NH_4OH solution. The solvent was evaporated, leaving a residue that was purified by flash chromatography (elution with hexane/EtOAc 9:1) to give 900 mg (90%) of product **8** as a colorless oil. ^{1}H NMR ($CDCl_3$) δ 2.27 (s, 1H), 3.45 (s, 3H), 3.43–3.47 (m, 1H), 3.54–3.55 (m, 2H), 3.63 (ABq, J=5.45 Hz, $\Delta\nu$=10.24 Hz, 1H), 3.74 (ABq, J=4.01 Hz, $\Delta\nu$=10.92 Hz, 1H), 3.80 (s, 3H), 4.47 (s, 2H), 6.88 (d, J=8.60 Hz, 2H), 7.25 (d, J=8.59 Hz, 2H); ^{13}C NMR ($CDCl_3$) δ 159.2, 129.9, 129.3, 113.8, 80.0, 73.1, 69.2, 62.4, 57.7, 55.2. FAB HRMS calcd for $C_{12}H_{18}O_4$ $(M)^+$ *m/z* 226.1205, found 226.1199.

3.1.2. 1-*O*-(1′-Octadecynyl)-2-*O*-methyl-3-*O*-(4′-methoxybenzyl)-*sn*-glycerol (9). A solution of diether glycerol derivative **8** (700 mg, 3.10 mmol) in 10 mL of THF was treated with KH (273 mg, 6.81 mmol) for 1 h. After the reaction mixture was cooled to −42°C, trichloroethylene (448 mg, 3.41 mmol) was added. The solution was warmed slowly to room temperature and stirred for 2 h. The resulting dark brown solution was cooled to −78°C, and treated with 2.8 mL (7.0 mmol) of *n*-BuLi (a 2.5 M solution in hexane). The solution was stirred for 1 h, then warmed to −42°C and stirred for 1 h. A solution of *n*-hexadecyl iodide (1.31 g, 3.72 mmol) in 10 mL of HMPA was added, and the reaction mixture was warmed to room temperature and stirred for 2 h. Methanol was then added to destroy excess KH and *n*-BuLi. The solution was diluted with 100 mL of Et_2O, washed with water (3×50 mL), and dried (Na_2SO_4). Evaporation of the solvents gave a brown residue that was purified by flash chromatography (elution with hexane/EtOAc 15:1 in the presence of 1% Et_3N by volume) to give 1.01 g (69%) of alkyne **9** as a light yellow oil. ^{1}H NMR ($CDCl_3$) δ 0.88 (t, J=6.72 Hz, 3H), 1.25 (s, 26H), 1.40–1.47 (m, 2H), 2.09 (t, J=6.94 Hz, 2H), 3.46 (s, 3H), 3.49 (ABq, J=5.27 Hz, $\Delta\nu$=9.29 Hz, 1H), 3.54 (q, J=4.90 Hz, 9.93 Hz, 1H), 3.63–3.67 (m, 1H), 3.81 (s, 3H), 4.03 (ABq, J=5.95 Hz, $\Delta\nu$=8.82 Hz, 1H), 4.09 (ABq, J=4.11 Hz, $\Delta\nu$=9.80 Hz, 1H), 4.48 (s, 2 H), 6.88 (d, J=8.56 Hz, 2H), 7.25 (d, J=8.16 Hz, 2H). ^{13}C NMR ($CDCl_3$) δ 159.3, 130.0, 129.3, 113.8, 89.5, 77.9, 77.5, 73.2, 68.3, 58.2, 55.3, 37.3, 31.9, 29.7, 29.7, 29.6, 29.4, 29.2, 28. 9, 22.7, 17.2, 14.1. FAB HRMS calcd for $C_{30}H_{49}O_4$ $(M-H)^+$ *m/z* 473.3631, found 473.3683.

3.1.3. 1-*O*-(1′-(*Z*)-Octadecenyl)-2-*O*-methyl-3-*O*-PMB-*sn*-glycerol (10). A solution of alkyne **9** (1.00 g, 2.11 mmol), Lindlar catalyst (100 mg), and quinoline (50 μL, 0.42 mmol) in 60 mL of hexane/EtOAc 1:1 was stirred under H_2 at 1 atm for 2 h. The solid was filtered through a silica gel pad that was washed with hexane/EtOAc 1:1. The filtrate was concentrated to give 1.00 g (100%) of *O*-alkenyl ether **10** as a light yellow oil. ^{1}H NMR ($CDCl_3$) δ 0.88 (t, J=6.80 Hz, 3H), 1.25 (s, 26H), 2.03 (q, J=6.96 Hz, 6.80 Hz, 2H), 3.46 (s, 3H), 3.47–3.57 (m, 3H), 3.82 (s, 3H), 3.76–3.87 (m, 2H), 4.31–4.36 (m, 1H), 4.48 (s, 2H), 5.92 (d, J=6.16 Hz, 1H), 6.87 (d, J=8.64 Hz, 2H), 7.26 (d, J=8.48 Hz, 2H); ^{13}C NMR ($CDCl_3$) δ 159.2, 145.0, 129.3, 113.8, 107.4, 79.3, 77.3, 73.6, 71.6, 68.9, 58.2, 55.3, 31.9, 29.8, 29.7, 29.6, 29.4, 29.3, 28.9, 23.9, 22.7, 14.1. FAB HRMS calcd for $C_{30}H_{52}O_4$ $(M)^+$ *m/z* 476.3866, found 476.3853.

3.1.4. 1-*O*-(1′-(*Z*)-Octadecenyl)-2-*O*-methyl-*sn*-glycerol (11). Twenty milliliters of liquid NH_3 was collected in a three-neck round-bottom flask by using a Dewar trap at −78°C. Sodium (100 mg, 4.35 mmol) was added, and the mixture was stirred for 1 h. A solution of *O*-alkenyl ether **10** (1.0 g, 2.10 mmol) in 20 mL of THF was added dropwise over a 3-min period. The reaction mixture was stirred for 1 h and warmed slowly to room temperature. MeOH was added to destroy the excess sodium. After the solution was diluted with 100 mL of Et_2O, water was added slowly, and the ammonia was removed by separation of the aqueous layer. The organic layer was dried (Na_2SO_4), and the solvents were evaporated. The residue was purified by flash chromatography (elution with hexane/EtOAc 3:1 with 1% NEt_3 by volume) to give 670 mg (90%) of alcohol **11** as a light yellow solid with a low melting point. ^{1}H NMR ($CDCl_3$) δ 0.88 (t, J=6.78 Hz, 3H), 1.25 (s, 26H), 2.05 (q, J=6.56 Hz, 6.28 Hz, 2H), 3.49 (s, 3H), 3.45–3.49 (m, 1H), 3.62 (ABq, J=5.66 Hz, $\Delta\nu$=10.24 Hz, 1H), 3.76 (ABq, J=3.77 Hz, $\Delta\nu$=11.03 Hz, 1H), 3.77–3.84 (m, 2H), 4.35–4.40 (m, 1H), 5.91, 5.93 (dt, J=1.32 Hz, 6.12 Hz, 1H, the *Z* vinyl proton), and a trace of *E* vinyl proton at δ 6.20; ^{13}C NMR ($CDCl_3$) δ 144.7, 107.8, 80.1, 71.2, 62.0, 58.1, 31.9, 29.7, 29.7, 29.7, 29.5, 29.4, 29.3, 23.9, 22.7, 14.1. FAB HRMS calcd for $C_{22}H_{45}O_3$ $(M+H)^+$ *m/z* 357.3369, found 357.3372.

3.1.5. 1-*O*-(1′-(*Z*)-Octadecenyl)-2-*O*-methyl-*sn*-glycero-3-*O*-phosphocholine (4). A solution of glycerol derivative **11** (200 mg, 0.561 mmol), pyridine (118 mg, 0.841 mmol), and 2-chloro-2-oxo-1,3,2-dioxaphospholane (104 mg, 0.73 mmol) in 8 mL of benzene was stirred overnight at 4°C. The solution was decanted to another flask and cooled to solidify. Lyophilization from benzene gave the cyclic phospholane intermediate as a white solid, which was immediately transferred to a pressure tube with 2 mL of benzene. After addition of 6 mL of MeCN, the solution was cooled to −20°C and 2 mL of NMe_3 were collected. The contents in the capped pressure tube were heated at 65°C for 48 h. The cooled solution was transferred onto a silica gel column, which was eluted with a gradient of $CHCl_3$/MeOH/H_2O (100:0:0, 80:20:0, 65:25:4) to give 200 mg (68%) of product **4** after lyophilization from benzene and filtration through a Cameo filter (Fisher Scientific Co.) to remove suspended silica gel; R_f 0.7 ($CHCl_3$/MeOH/H_2O 65:25:4); $[\alpha]^{25}_D$-1.97° (*c* 0.64, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 5.92 (d, J=6.20 Hz, 1H), 4.30–4.35 (m, 3H), 3.82–3.95 (m, 5H), 3.76 (ABq, J=6.10 Hz, $\Delta\nu$=9.25 Hz, 1H), 3.51–3.57 (m, 1H), 3.44 (s, 3H), 3.39 (s, 9H), 2.02 (q, J=6.76 Hz, 6.84 Hz, 2H), 1.25 (s, 28H), 0.88 (t, J=6.44 Hz, 3H); ^{13}C NMR ($CDCl_3$) δ 144.9, 107.4, 79.5 (d, J_{C-P}=8.0 Hz), 71.7, 66.3 (d, J_{C-P}=6.0 Hz), 64.2 (d, J_{C-P}=5.0 Hz, 59.4 (d, J_{C-P}=3.0 Hz), 58.1, 54.4, 31.9, 29.9, 29.7, 29.7, 29.7, 29.4, 29.4, 24.0, 22.7, 14.1. Anal. calcd for $C_{27}H_{56}O_6NP{\cdot}2.5H_2O$: C,

57.23; H, 10.85; N, 2.47; P, 5.47. Found: C, 57.07; H, 10.91; N, 2.45; P, 4.75.

3.2. Effect of compounds 2 and 4 on cell proliferation

The effects of the compounds on the 48 h increase in cell numbers were assessed using procedures described previously.[17]

3.3. Expression of phospholipid growth factor receptor mRNA in the cell lines

For RNA isolation, cells were harvested into RNAlater™ (Ambion, Austin, TX). RNA was digested with RNase-free DNase (Stratagene, San Diego, CA) to remove genomic DNA contamination, and cDNA was obtained by reverse transcription using the SuperScript Preamplification System (Gibco-BRL, Gaithersburg, MD). The oligonucleotide primers, derived from the human orthologs of the LPA receptor sequences, were as follows: Edg-2 forward primer 5′-65agatctgaccagccgactcac-3′, reverse primer 5′-gttggccat caagtaataaata422-3′; Edg-4 forward primer 5′-634ctgctcagc cgctcctatttg-3′, reverse primer 5′-aggagcacccacaagtcatcag 1185-3′; Edg-7 forward primer 5′-91agcaacactgatactgtcgatg -3′, reverse primer 5′-gcatcctcatgattgacatgtg446-3′; psp24 forward primer 5′-320ctgcatcatcgtgt accagag, reverse primer 5′-acgaactctatgcaggcctcgc1184-3′, and β-actin forward primer 5′-36tcaccatggatgatgatatcgc, reverse primer 5′-cgtgctcgatggggtacttca251-3′. After a 1-min predenaturation at 94°C, 30 amplification cycles were preformed, each consisting of a 10-s denaturation at 94°C, annealing for 30 s at 60°C, and elongation for 1 min at 72°C. A final elongation was done for 5 min at 72°C.

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