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Alkoxymethylenephosphonate Analogues of (Lyso)phosphatidic Acid Stimulate Signaling Networks Coupled to the LPA_2 Receptor

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An efficient stereocontrolled synthesis afforded alkoxymethylenephosphonate (MP) analogues of lysophosphatidic acid (LPA) and phosphatidic acid (PA). The pharmacological properties of MP-LPA and MP-PA analogues were characterized for LPA receptor subtype-specific agonist and antagonist activity using Ca^{2+}-mobilization assays in RH7777 cells expressing the individual LPA_1–LPA_3 receptors and CHO cells expressing LPA_4. In addition, activation of a PPARγ reporter gene construct expressed in CV-1 cells was assessed. These metabolically stabilized LPA analogues exhibited an unexpected pattern of partial agonist/antagonist activity for the LPA G-protein-coupled receptor family and the intracellular LPA receptor PPARγ. Analogues were compared with 18:1 LPA for activation of downstream signaling in HT-29 colon cancer cells, which exclusively express LPA_2, and both SKOV3 and OVCAR3 ovarian cancer cells, which express LPA_1, LPA_2, and LPA_3. Unexpectedly, reverse phase protein arrays showed that four MP-LPA and MP-PA analogues selectively activated downstream signaling in HT-29 cells with greater potency than LPA. In particular, the oleoyl MP-LPA analogue strongly promoted phosphorylation and activation of AKT, MEK, and pS6 in HT-29 cells in a concentration-dependent manner. In contrast, the four MP-LPA and MP-PA analogues were equipotent with LPA for pathway activation in the SKOV3 and OVCAR3 cells. Taken together, these results suggest that the MP analogues may selectively activate signaling via the LPA_2 receptor subtype, while simultaneously suppressing signaling through the LPA_1 and LPA_3 subtypes.

Introduction

Among the rich variety of phospholipids involved in cell signaling, lysophosphatidic acid (LPA) and phosphatidic acid (PA) play very important roles. LPA (1- or 2-radyl-*sn*-glycerol 3-phosphate) is a deceptively simple ligand that elicits a rich palette of biological responses, including platelet aggregation, promotion of cell survival, and cell migration.[1,2] LPA also appears to be involved in the pathophysiology of cancer[3,4] and infertility.[5,6] Elevated LPA concentrations are observed in the plasma of patients with multiple myeloma and in the ascitic fluid of patients with ovarian cancer, where it is a marker of tumor progression.[7–9] Most of these cellular events are transduced via three G-protein-coupled receptors (GPCRs): LPA_1, LPA_2, LPA_3, also known respectively as EDG2, EDG4, and EDG7.[10] Recently two non-EDG LPA GPCRs, LPA_4/GPR23[11] and LPA_5/GPR92,[12,13] as well as the nuclear transcription factor PPARγ, have been reported to interact with LPA.[11–14] Each receptor has a unique structure–activity relationship for acyl groups, backbone, and phosphate analogues.[15,16] One of the catabolic pathways for termination of LPA signaling is via the LPA-specific acyltransferases, which produce PA. The action of phospholipase D on phosphatidylcholine and other phospholipids is another important source of PA, which regulates phosphoinositide metabolism and plays key roles in cell growth and vesicular trafficking of proteins. The balance between LPA and PA alters membrane curvature and thus facilitates the formation of vesicles and affects cellular signaling.[17,18] There is evidence that PA analogues with short- and long-chain fatty acids or fatty alcohols also activate the EDG receptors.[19,20]

The design and synthesis of LPA and PA analogues has generated a limited number of ligands with subtype selectivity.[14,21–24] However, whereas analogues with relative selectivity for LPA_1 and LPA_3 have been identified, good selectivity for LPA_2 is uncommon. Nonetheless, computational studies combined with mutational analysis of LPA receptors has begun to provide a predictive model for isoform selectivity.[20,25] We previously reported the synthesis of several LPA and PA analogues[26] with partial selectivity for individual LPA GPCRs and PPARγ.[14,27–33] Among these, **2** was identified as selective PPARγ agonists, and the α-fluoromethylene phosphonate **3**

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emerged as a potent LPA_3-selective agonist (Figure 1). More recently, we described a series of α-halomethylene and α-hydroxymethylene LPA phosphonate analogues such as α-bromo compound **4**, which acted as a pan-LPA GPCR receptor antagonist and a potent autotaxin inhibitor.[34] Thus far, none of the phosphonate analogues of LPA or PA have retained the 3-oxygen functionality of the glyceryl backbone; instead, that oxygen atom has been replaced with methylene, fluoromethylene, and difluoromethylene.[26] We now describe a synthetic route that provides access to a stabilized phosphonate that retains the glyceryl 3-oxygen as well as the dianionic head group. In this modification, a methylene bridge was inserted between the oxygen atom of the glycerol skeleton and the phosphate head group. An analogous approach was previously exploited to generate alkoxymethylene phosphonate-containing geranylgeranyl protein transferase inhibitors[35] and antiviral drugs,[36] including anti-HIV phosphorylated nucleoside analogues.[37,38] Recently, we also prepared phosphatase-resistant analogues of the phosphoinositides PtdIns(3)P,[39] PtdIns(5)P,[40] and PtdIns(3,4,5)P_3,[41] and we demonstrated long-lasting agonist effects of the 3-phosphatase-resistant MP analogue of PtdIns-(3,4,5)P_3. We sought to extend this concept to the production of MP analogues of LPA and PA. Herein we describe an efficient synthesis of the *sn*-2-OH alkoxymethylenephosphonate (MP) analogues of LPA, and two related bis-acylated PA analogues. We show the pharmacological properties of these ligands at LPA_{1-3} GPCRs in the McArtl rat hepatoma cell line RH7777, LPA_4 in CHO cells, and a PPARγ reporter gene expressed in CV-1 cells. In addition, we summarize the responses of three mammalian cancer cell lines (SKOV3, OVCAR3, and HT-29) to these analogues using a novel reverse phase protein array (RPPA) technique.

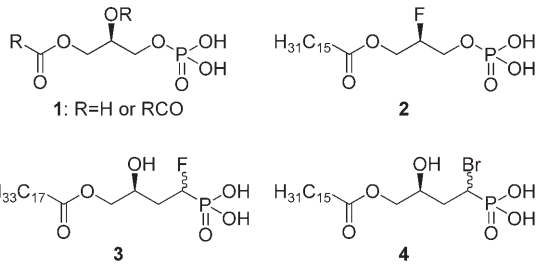


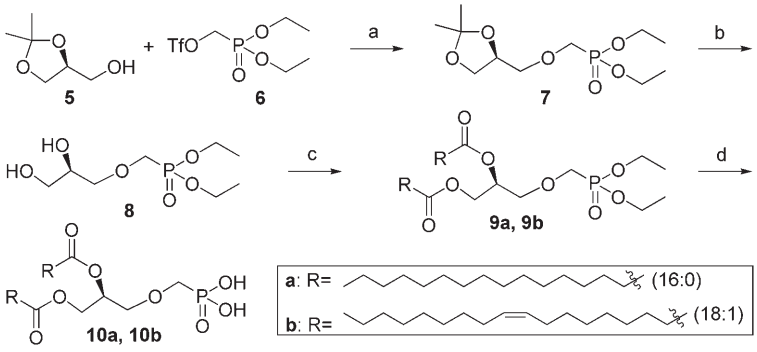
Figure 1. Structures of parent LPA and PA **1**, PPARγ-selective agonist **2**, potent LPA_3-selective analogue **3**, and potent pan-LPA GPCR antagonist and ATX inhibitor **4**.

Results

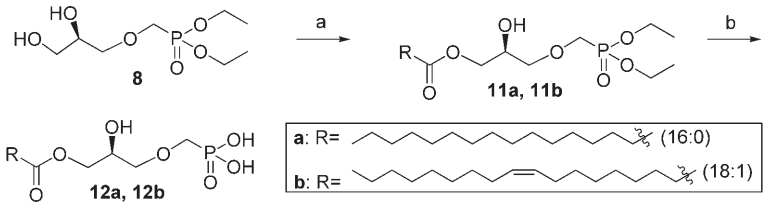
Chemical synthesis

Commercially available (*S*)-isopropylideneglycerol (**5**) was treated with *n*BuLi at −78 °C,[35] and the resulting anion was treated with triflate **6**[39,41,42] to give the protected phosphonate **7** (Scheme 1). The isopropylidene group was removed with an acidic ion-exchange resin[43,44] to produce the intermediate diol **8**. Next, esterification with either (**a**) palmitic or (**b**) oleic acid using ethyl diaminomethylpropyl carbodiimide (EDC) and 4-(*N*,*N*-dimethyl)aminopyridine (DMAP) led to the desired bis-acylated MP-PA analogues **9a** and **9b** in high yields. Deprotection of the phosphonate in the presence of a significant excess of bromotrimethylsilane (TMSBr)[45] proceeded quantitatively to give the homogenous MP-PA analogues **10a** and **10b**.

Carbodiimide-promoted esterification was also used to prepare the LPA analogues (Scheme 2). Compounds **11a** and **11b** were obtained in good yields along with small amounts of *sn*-2 acylated and PA derivative byproducts, which were readily removed by chromatography. The main advantage of an EDC-catalyzed esterification was the ability to remove the urea byproduct during aqueous workup. Other esterification methods required several purifications with correspondingly lower yields. Finally, treatment of each ester with bis(trimethylsilyl)acetamide (BSA)[46] and TMSBr for 3.5 h and subsequent addition of 10% aqueous methanol resulted in deprotection of the ethyl groups. The solutions of the crude MP-LPA compounds



Scheme 1. Synthesis of MP-PA analogues **10a** (16:0) and **10b** (18:1): a) *n*BuLi, Et_2O, −78 °C→0 °C, 62%; b) Dowex [H^+], CH_3OH, 84%; c) RCOOH, EDC, DMAP, CH_2Cl_2, room temperature, 85–87%; d) TMSBr, CH_2Cl_2/CH_3CN, room temperature, overnight, quant.



Scheme 2. Synthesis of *sn*-2-OH MP-LPA analogues **12a** (16:0) and **12b** (18:1): a) RCOOH, EDC, DMAP, CH_2Cl_2, room temperature, 53–68%; b) BSA, TMSBr, CH_3CN, 3.5 h, room temperature, 73–88%.

12a and **12b** in CH_2Cl_2 were washed with a 1:1 mixture of water/brine and dried to provide the homogenous analogues.

Activation of LPA GPCRs and PPARγ

The ligand properties of the compounds were evaluated for the activation of human LPA_1, LPA_2, and LPA_3 individually expressed in RH7777 cells and LPA_4 expressed in CHO cells by measuring Ca^{2+} mobilization. The activation of the intracellular LPA receptor PPARγ was monitored in CV-1 cells transfected with PPARγ and its reporter gene construct PPRE-ACox-luc. Table 1 summarizes the agonist and antagonist activities of the PA analogues **10** and LPA analogues **12**. In these experiments, RH7777 cells, which are intrinsically unresponsive to LPA, were transfected with the appropriate receptor.[21,47] Unexpectedly, the bis-acylated MP-PA analogues **10** acted as low micromolar to submicromolar inhibitors of the three canonical receptors LPA_1, LPA_2, and LPA_3, with the highest potency for the LPA_1 receptor. More in line with expectations, the "methylene-extended" MP-LPA analogues **12** were partial or full agonists for LPA_1, LPA_2, and LPA_4, but curiously showed modest antagonist activity towards the LPA_3 receptor. Figure 2 illustrates the effects of the MP analogues on PPARγ activation. The data show that the unsaturated PA analogue **10b** (18:1) and the LPA analogues **12a** (16:0) and **12b** (18:1) induced modest, but statistically significant, luciferase expression through the acyl-CoA oxidase PPRE reporter. In contrast, the saturated PA analogue **10a** (16:0) did not activate this PPARγ reporter construct.

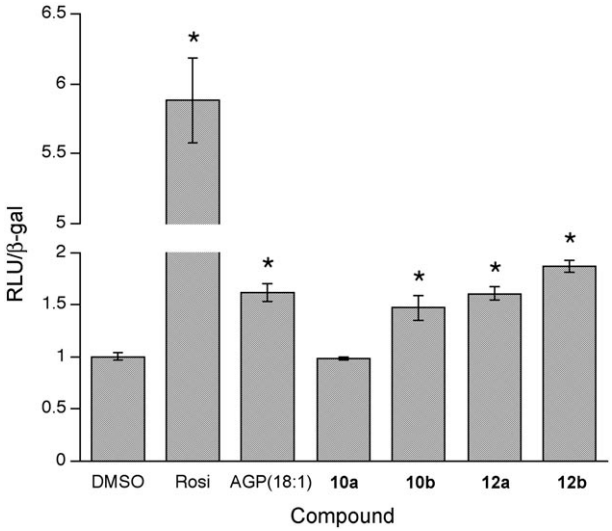


Figure 2. Activation of the PPRE-ACox-luc reporter gene construct by the novel LPA analogues. The thiazolidinedione compound rosiglitazone (Rosi), octadecenyl glycerophosphate (AGP 18:1), or the test compounds were each applied at 10 μM for 20 h to CV-1 cells transfected with the reporter gene construct. The results represent the fold stimulation in luciferase expression normalized to the vehicle ($n = 5 \pm$ SD, $^*p < 0.01$).

Activation of endogenous LPA signaling pathways in cancer cells

The effects on downstream signaling through the LPA receptor subtypes by both MP-LPA and MP-PA analogues was evaluated in HT-29, OVCAR3, and SKOV3 cancer cell lines using an emerging technology, the reverse phase protein array (RPPA).[48–50] In preliminary studies, each of the four analogues increased phosphorylation of AKT (T308), AKT (S473), EGFR (Y1173), GSK3 (S21/9), JNK (T183/Y185), p38 (T180/Y182), MEK (S217/221), and MAPK (T202/Y204) in all three cell lines. HT-29 cells appeared to be more sensitive to the MP-PA and MP-LPA analogues, with the level of phosphorylation of signaling molecules nearly double that of 18:1 LPA (10 μM) stimulated HT-29 cells for many downstream kinase substrates. The initial RPPA studies were then repeated in triplicate for the HT-29 cells with a focus on 18:1 LPA, the palmitoyl MP-LPA analogue **12a**, and the oleoyl MP-LPA analogue **12b**. The results for relative protein phosphorylation of 11 selected signaling substrates in HT-29 cells are shown in Figure 3. Many of the downstream substrates such as MAPK or GSK3 did not show a clear dose–response effect for phosphorylation induced by LPA or either of the MP-LPA analogues. In contrast, the oleoyl MP-LPA analogue **12b** showed potent phosphorylation and activation of target substrates such as AKT (S473), MEK (S217/221), and S6 (S235/

Table 1. Pharmacological characterization at LPA GPCR and PPARγ-PPRE luciferase reporter gene assay.

Compd	LPA_1		LPA_2		LPA_3		LPA_4		PPARγ
	EC_{50} [nM] (E_{max})[a]	IC_{50} [nM] (Inhib. [%])[c]	EC_{50} [nM] (E_{max})	IC_{50} [nM] (Inhib. [%])[d]	EC_{50} [nM] (E_{max})	IC_{50} [nM] (Inhib. [%])[c]	EC_{50} [nM] (E_{max})	IC_{50} [nM] (Inhib. [%])	
10a	NE[b]	518 ± 340 (63.1 ± 12.1)	NE	> 7380 (58.8 ± 5.6)[e]	NE	> 3610 (68.8 ± 7.4)[e]	NE	NE	NE
10b	NE	1520 ± 494 (60.0 ± 7.5)	NE	2680 ± 880 (82.9 ± 2.5)	NE	> 7540 (71.4 ± 3.8)[e]	NE	NE	Agonist
12a	5290 ± 566 (25.6 ± 1.1)	NE	> 1010 (30.6 ± 6.4)[e]	NE	NE	> 3060 (91.2 ± 2.3)[e]	4340 ± 98.5 (66.5 ± 0.7)	NE	Agonist
12b	> 4000 (44.4 ± 8.5)[e]	NE	258 ± 142 (53.2 ± 5.8)	NE	NE	1360 ± 749 (50.3 ± 3.0)	> 1790 (122 ± 2.1)[e]	NE	Agonist

[a] E_{max} = (maximal efficacy of compound)/(maximal efficacy of LPA 18:1) × 100. [b] NE = no effect was detected at the highest concentration (30 μM) tested. [c] Percent maximal inhibition of the response to 200 nM LPA 18:1. [d] Percent maximal inhibition of the response to 10 nM LPA 18:1. [e] E_{max} at 30 μM.

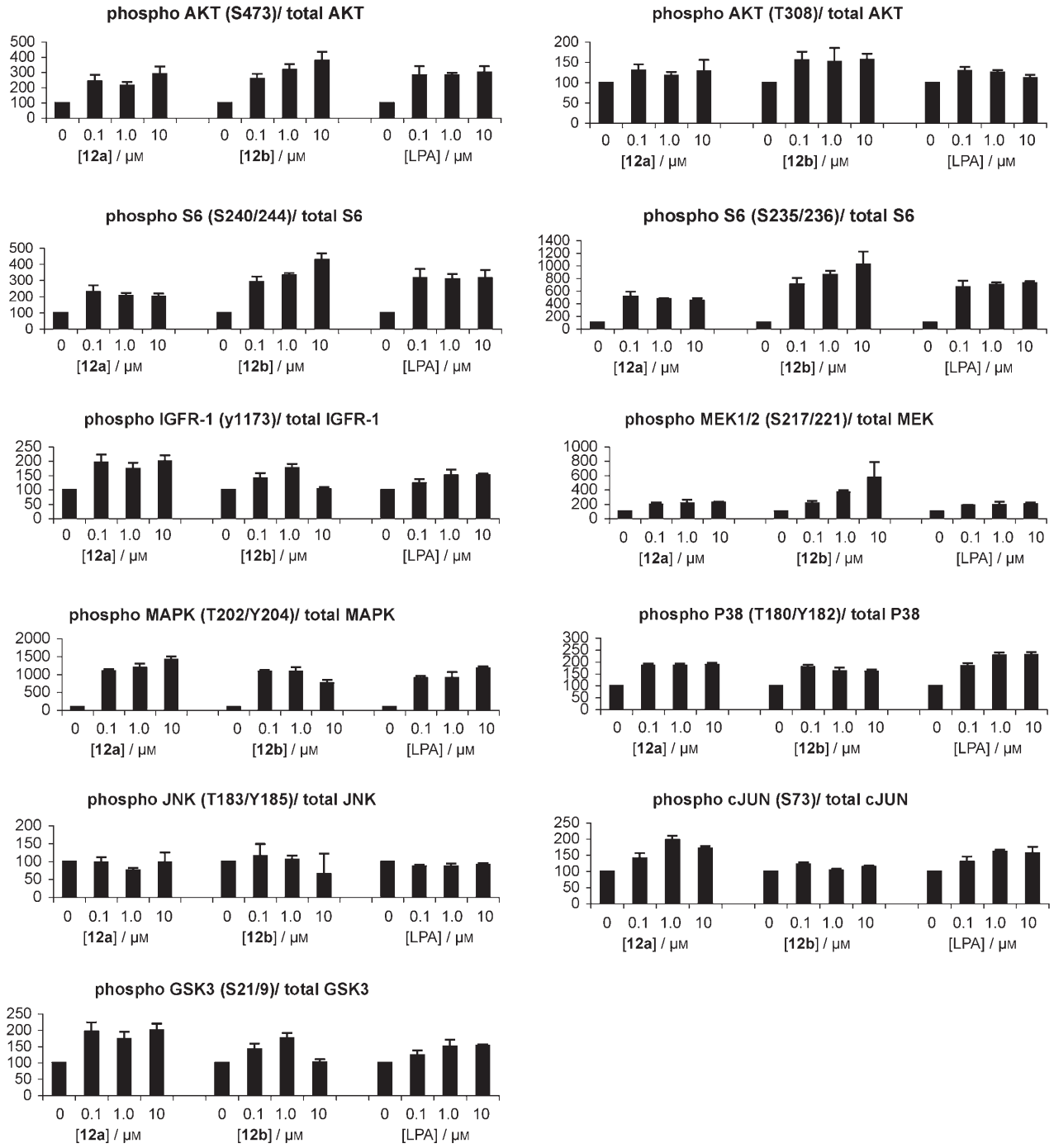


Figure 3. RPPA results showing the effects of 0, 0.1, 1.0, and 10 μM LPA, palmitoyl MP-LPA analogue **12a**, and oleoyl MP-LPA analogue **12b** on cell signaling in HT-29 cells for 11 substrate/product pairs. Bars represent intensity readings analyzed by MicroVigene and indicate phosphorylation relative to protein loaded in the microarray as determined by immunodetection (*y*-axes: fold increase over control × 100; $n = 3 \pm$ SD). Refer to the Experimental Section for a full description of the RPPA.

236). This downstream signaling data, in which the output of multiple interactions is observed as the readout, could not be determined or anticipated from the single-GPCR transfected cellular assays, and thus adds an important new dimension to the understanding of the mode of action of LPA and its analogues in cell physiology. Furthermore, the timescales of the calcium translocation assays (seconds) and the protein phosphorylation assays (tens of minutes), likely sample a different spectrum of cellular responses, including different pharmacokinetics and regulatory loops.

In contrast to the unexpected potency on HT-29 cells, statistical analysis showed that the four MP analogues **10a**, **10b**, **12a**, and **12b** are equipotent with LPA for pathway activation in the SKOV3 and OVCAR3 cells (Figure 4). In addition, the ex-

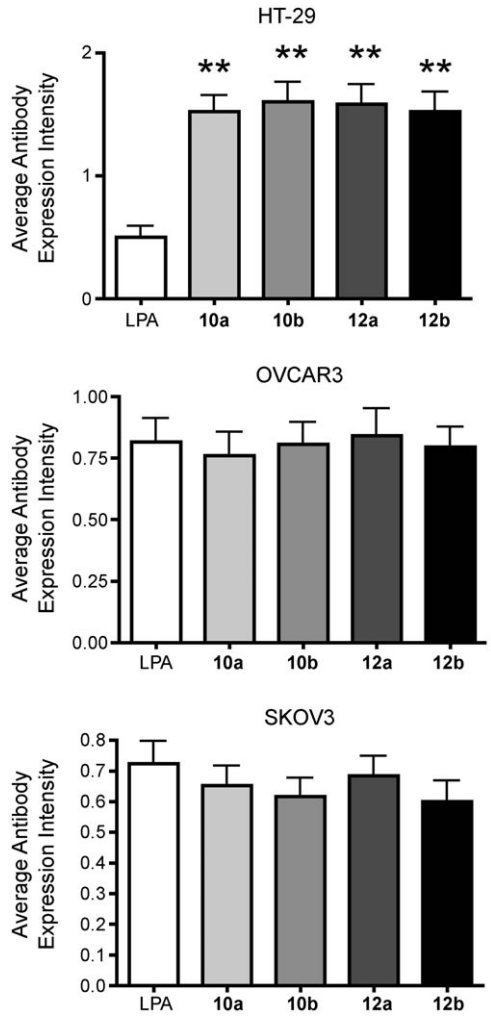


Figure 4. Statistical analysis of RPPA results. The difference between RPPA expression intensities at 0 and 10 μM of LPA or an MP-PA analogue **10** or an MP-LPA analogue **12** was calculated for each antibody tested. The data compares average antibody expression intensity for each agonist versus LPA as the control. Asterisks indicate statistical significance ($^{**}p < 0.01$) determined by ANOVA followed by Dunnett's multiple comparison post test.

pression of LPA_2 and the absence of LPA_1, LPA_3, LPA_4, and LPA_5 was confirmed by RT-PCR in HT-29 cells (Figure 5). Taken together, these results suggest that the MP-LPA analogues potently activate signaling pathways in the LPA_2-dominant HT-29 cells than in other cell types.

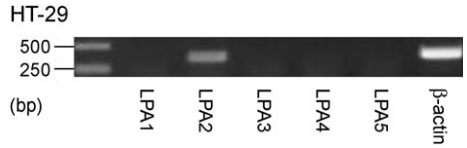


Figure 5. RT-PCR analysis of HT-29 cells illustrating that LPA_2 is only one of five known LPA GPCRs expressed in this cell line.

Discussion

We have characterized the synthesis and pharmacological properties of four metabolically stabilized LPA analogues, two related bis-acylated (MP-PA) analogues, and the *sn*-2-OH alkoxymethylene phosphonate (MP-LPA) analogues. Overall, our results suggest the MP analogues selectively activate signaling via the LPA_2 receptor subtype, while simultaneously suppressing signaling through the LPA_1 and LPA_3 subtypes. Our groups have previously described the design and synthesis of a variety of ligands that show isoform selectivity for LPA receptors.[20,26,31,33,51] Nonetheless, we had not examined phosphonate analogues that retain the important 3-oxygen functionality of the glyceryl backbone. The compounds described herein possess a stabilized phosphonate, the glyceryl 3-oxygen, and the dianionic head group.

Prior to performing the RPPA studies, preliminary studies were carried out to compare the activity of 18:1 LPA with MP-LPA analogues **12a** and **12b** and MP-PA analogues **10a** and **10b** in HT-29 (colon cancer), OVCAR3 (ovarian cancer), SKOV3 (ovarian cancer), MDA-MB-231 (breast cancer) and MDA-MB-468 (breast cancer) cell lines. These cell lines were selected based on their responses to LPA and collective diversity of LPA receptor expression. HT-29 cells predominantly express LPA_2, OVCAR3 cells express $LPA_3 > LPA_2 > LPA_1 \geq LPA_4$, SKOV3 cells express $LPA_2 > LPA_1 \gg LPA_3 > LPA_4$, and MDA-MB-231 cells express $LPA_1 \gg > LPA_2$.[52] In the context of the results with LPA_2-expressing HT-29 cells, it is important to recognize that LPA_2 is overexpressed and regulates migration in a variety of gastrointestinal cancers[53–55] and breast ductal carcinomas.[56] LPA_2 is important, but less important than LPA_1, in Rho-dependent chemotaxis in breast carcinoma cells[57] and mediates mitogenic signals in human colon cancer cells.[58]

The results from cell proliferation assays show that the two MP-LPA analogues **12a** and **12b** stimulated proliferation in HT-29 cells, and both were more active than 18:1 LPA (data not shown). In contrast, in OVCAR3 and SKOV3 cells, both MP-LPA analogues **12a** and **12b** were equivalent in activity to 18:1 LPA (data not shown). No stimulation of cell proliferation was observed for the MP-LPA analogues in either MB-231 or MB-468 breast cells. Since HT-29 cells are only able to signal via the LPA_2 subtype, the MP-LPA analogues appear to be LPA_2-selective. The MP-PA analogues **10a** and **10b** stimulated cell proliferation at low doses (0.1–1.0 μM) in most cell lines, but exhibited cytotoxicity at 10 μM (data not shown).

These synthetic analogues exhibited a biologically surprising pattern showing both partial agonist and antagonist activity towards the LPA G-protein-coupled receptors and PPARγ, the intracellular LPA receptor. The major findings that support this conclusion come from the RPPA data. These results demonstrate an unexpected phenomenon whereby all four MP-LPA and MP-PA analogues are more potent than LPA in promoting the activation of AKT, GSK3, JNK, p38, and ERK in HT-29 colon cancer cells that predominantly express the LPA_2 receptor. The four MP analogues are equipotent with LPA for pathway activation in both SKOV3 and OVCAR3 cells. These ovarian cancer cells express combinations of LPA receptors, likely resulting in

both signaling activation and suppression from the analogues. The MP analogues could be useful to selectively activate the LPA_2 receptor in cell types expressing multiple receptors. Furthermore, the present results suggest a strong cross-modulatory effect between LPA GPCRs when expressed simultaneously in the same cell, as indicated by the increased signaling responses elicited by the selective agonist/antagonist analogue versus the natural pan-agonist. However, this observation will have to be examined in future experiments using a combination of pharmacological and receptor add-back studies. Taken together, these results suggest that the LPA_2-dominant HT-29 cells more potently activate signaling pathways in response to both MP-PA and MP-LPA analogues than other cell types.

Experimental Section

General synthetic procedures. Chemicals were obtained from Aldrich and Acros and were used without further purification. Solvents were purchased anhydrous (Et_2O and THF) or reagent-grade, and distilled before use: CH_2Cl_2 was distilled from CaH_2. Reactions requiring anhydrous conditions were carried out in oven-dried glassware (2 h, 120 °C) under inert atmosphere (N_2 or Ar) unless otherwise indicated. Concentration in vacuo refers to the use of rotary evaporator for solvent removal, and purification on SiO_2 refers to flash chromatography (FC) on silica gel. NMR spectra were recorded at 400 MHz (^1H), 101 MHz (^{13}C), or 162 MHz (^{31}P) at ambient temperature. Chemical shifts are reported relative to those of internal chloroform peaks ($\delta_H = 7.24$ ppm), and ($\delta_C = 77.0$ ppm) and to [D_6]DMSO peaks ($\delta_H = 2.49$ ppm) and ($\delta_C = 39.5$ ppm). Optical rotations were obtained at ambient temperature. Symbols: s, singlet; bm, broad multiple; bs, broad singlet; dd, doublet of doublets; m, multiplet; p, quintuplet; q, quartet; t, triplet. Coupling constants (J) are all reported in Hz.

Diethyl phosphonomethyltriflate (6). A mixture of diethyl hydroxymethylphosphonate (3.26 g, 19.4 mmol) and NaH (0.6 g, 25.2 mmol) (washed three times with dry hexanes) in anhydrous Et_2O (30 mL) was stirred for 4 h at room temperature. This suspension of sodium alkoxide was added dropwise over 40 min to a cooled (−78 °C) solution of CF_3SO_2Cl (30 mL, 29.1 mmol) in Et_2O (30 mL). The resulting solution was stirred for an additional 10 min and then slowly warmed to −5 °C. After filtration to remove excess NaH, the filtrate was diluted with CH_2Cl_2, extracted with saturated $NaHCO_3$, then dried (Na_2SO_4) and concentrated to afford an oil. Because the crude compound was contaminated with bis(diethyl phosphonomethyl) ether, the mixture was easily separated by partitioning between Et_2O/H_2O, affording pure triflate in 44% yield (8.6 mmol, 2.58 g). ^1H NMR ($CDCl_3$): $\delta = 4.52$ (d, 2H, $J = 9.2$ Hz), 4.18–4.10 (m, 4H), 1.28 ppm (t, 6H, $J = 7.2$ Hz); ^{13}C NMR ($CDCl_3$): $\delta = 118.4$ (q, $J = 321.1$ Hz), 67.2, 65.5, 63.7 (d, $J = 6.8$ Hz), 16.1 ppm (d, $J = 5.3$ Hz); ^{31}P NMR ($CDCl_3$): $\delta = 13.2$ ppm; ^{19}F NMR ($CDCl_3$): $\delta = -74.6$ ppm; MS (CI) m/z 169.1 ([$M-SO_2CF_3+1$], 100), 301.1 ([$M+1$], 44); HRMS (CI) for $C_6H_{13}F_3O_6PS$, found: 301.0138, calcd: 301.0123.

Diethyl 1,2-(*R*)-isopropylidene-*sn*-glycerol-3-methylphosphonate (7). *n*BuLi (1.46 mL 3.66 mmol,) was added dropwise over 5 min to a stirred solution of (*S*)-isopropylideneglycerol (0.433 mL, 3.66 mmol) in anhydrous THF (30 mL) cooled to −78 °C under an argon atmosphere. After stirring for 1 h at −78 °C the mixture was treated dropwise with a solution of diethyl phosphonomethyltriflate (3.66 mmol, 1 g) in anhydrous THF (10 mL). The reaction mixture was stirred for another 1 h at −78 °C, and was then allowed to warm to 0 °C for 2 h. The reaction was quenched by the addition of saturated aq NH_4Cl and extracted with Et_2O. The organic phase was dried and evaporated under reduced pressure. The resulting residue was purified by FC with hexanes/acetone 1:1 to give compound **7** in 62% yield (2.3 mmol, 0.65 g). $R_f = 0.36$ (hexanes/acetone 1:1), $[\alpha]_D^{20} = +12.9$ ($c = 1.17$, $CHCl_3$); ^1H NMR ($CDCl_3$): $\delta = 4.81$ (p, 1H, $J = 5.6$ Hz), 4.11–4.04 (m, 4H), 3.97–3.93 (m, 1H), 3.83–3.72 (m, 2H), 3.68–3.64 (m, 1H), 3.56 (d, 2H, $J = 6$), 1.31 (s, 3H), 1.27–1.23 ppm (overlapping signals, 9H); ^{13}C NMR ($CDCl_3$): $\delta = 109.6$, 74.7, 74.0, 74.9, 66.6, 64.9, 62.5 (d, $J = 6.2$ Hz), 26.8, 25.5, 16.6 ppm (d, $J = 6.2$ Hz); ^{31}P NMR ($CDCl_3$): $\delta = 22.1$ ppm (decoupled); LRMS (CI) m/z 283.1 ([$M+1$], 100); HRMS (CI) for $C_{11}H_{24}O_6P$, found: 283.1322, calcd: 283.1311.

Diethyl (*R*)-1,2-dihydroxy-*sn*-glycerol-3-methylphosphonate (8). Compound **7** (640 mg, 2.27 mmol) was dissolved in methanol (20 mL) and treated with an excess of Dowex [H^+] (640 mg). The reaction mixture was stirred for 3 h at room temperature, filtered, concentrated, and purified by FC on SiO_2 using a mixture of CH_2Cl_2/CH_3OH 19:1. Pure compound **8** was obtained as a colorless oil in 84% yield (464 mg, 1.92 mmol). $R_f = 0.45$ (CH_2Cl_2/CH_3OH 9:1); $[\alpha]_D^{20} = -2.5$ ($c = 1.07$, $CHCl_3$); ^1H NMR ($CDCl_3$): $\delta = 4.17–4.06$ (overlapping signals, 5H), 3.85–3.80 (overlapping signals, m, 3H), 3.66–3.48 (m, 5H), 1.31 ppm (t, 6H, $J = 7.2$ Hz); ^{13}C NMR ($CDCl_3$): $\delta = 72.6$ (d, $J = 9.9$ Hz), 70.6, 66.1, 64.5, 63.3, 62.5 (m), 16.3 ppm (d, $J = 6.2$ Hz); ^{31}P NMR ($CDCl_3$): $\delta = 22.8$ (decoupled); LRMS (CI) m/z 243.1 ([$M+1$], 100); HRMS (CI) for $C_8H_{20}O_6P$, found: 243.1001, calcd: 243.0998.

Diethyl (*R*)-1,2-dipalmitoylpropane-3-methylphosphonate ester (9a). To a solution of **8** (50 mg, 0.21 mmol) in anhydrous CH_2Cl_2 (2 mL) was added palmitic acid (107 mg, 0.42 mmol) followed by the addition of EDC (112 mg, 0.59 mmol) and DMAP (20 mg, 0.17 mmol) as a mixture in CH_2Cl_2 (0.5 mL). The reaction was stirred for 5 h at room temperature under Ar. The reaction was diluted with CH_2Cl_2 and washed with H_2O and brine. It was then dried with Na_2SO_4, filtered, and concentrated in vacuo. The resulting residue was purified chromatographically with hexanes/acetone 4:1 to produce the desired compound as a white solid in 87% yield (132 mg, 0.18 mmol). $R_f = 0.38$ (hexanes/acetone 7:3); $[\alpha]_D^{20} = +4.06$ ($c = 0.69$, $CHCl_3$); ^1H NMR ($CDCl_3$): $\delta = 5.19–5.14$ (m, 1H), 4.29–4.26 (m, 1H), 4.15–4.07 (overlapping signals, m, 5H), 3.82–3.71 (m, 2H), 3.70–3.64 (m, 2H), 2.27–2.22 (m, 4H), 1.57–1.53 (m, 4H), 1.31–1.14 (overlapping signals, m, 54H), 0.82 ppm (t, 6H, $J = 7.2$ Hz); ^{13}C NMR ($CDCl_3$): $\delta = 173.2$, 172.9, 71.4, 71.3, 69.6, 66.3, 64.7, 62.4 (m), 34.2, 34.0, 31.8, 29.6, 29.6, 29.5, 29.4, 29.3, 29.2, 29.0, 29.0, 24.8, 24.7, 22.6, 16.4, 16.4, 14.0 ppm; ^{31}P NMR ($CDCl_3$): $\delta = 21.5$ ppm (decoupled); LRMS (CI) m/z 719 ([$M+1$], 24), 257.2 ([$H_{31}C_{15}COOH+1$], 74), 137.1 ([$P(O)(OC_2H_5)_2$], 100); HRMS (CI) for $C_{40}H_{80}O_8P$, found: 719.5591, calcd: 719.5588.

Diethyl (*R*)-1,2-dioleoylpropane-3-methylphosphonate ester (9b). The method for **9a** was employed, but with oleic acid instead of palmitic acid. Compound **9b** was obtained as a colorless oil in 85% yield (130 mg, 0.18 mmol). $R_f = 0.38$ (hexanes/acetone 7:3); $[\alpha]_D^{20} = +3.68$ ($c = 0.76$, $CHCl_3$); ^1H NMR ($CDCl_3$): $\delta = 5.36–5.27$ (m, 4H), 5.21–5.16 (m, 1H), 4.30 (dd, 1H, $J = 4.0$, 11.6 Hz), 4.17–4.09 (overlapping signals, m, 5H), 3.84–3.74 (m, 2H), 3.72–3.66 (m, 2H), 2.30–2.25 (m, 4H), 2.00–1.95 (m, 8H), 1.64–1.51 (m, 4H), 1.34–1.22 (overlapping signals, m, 46H), 0.84 ppm (t, 6H, $J = 6.8$ Hz); ^{13}C NMR ($CDCl_3$): $\delta = 173.3$, 172.9, 129.9, 129.7, 71.3, 69.7, 66.4, 64.7, 62.4 (m), 34.2, 34.0, 31.9, 30.9, 29.7, 29.7, 29.5, 29.3, 29.2, 29.1, 29.0, 27.2, 27.1, 24.8, 22.6, 16.5, 16.4, 14.1 ppm; ^{31}P NMR ($CDCl_3$): $\delta = 21.5$ ppm; LRMS (CI) m/z 771 ([$M+1$], 4.98), 311 ([$H_{33}C_{17}COOC_2H_5+1$], 100); HRMS (CI) for $C_{44}H_{84}O_8P$, found: 771.5909, calcd: 771.5904.

(*R*)-1,2-Dipalmitoylpropane-3-methylphosphonate ester (10a). Compound **9a** (86 mg, 0.12 mmol) was dried overnight under high vacuum before it was dissolved in a mixture of anhydrous CH_2Cl_2/CH_3CN (4 mL, 1:3, *v*/*v*). TMSBr (0.9 mL) was added under N_2. The reaction was stirred overnight at room temperature, then the solvents were removed under reduced pressure. The residue was dried for 2 h under high vacuum and was then dissolved in 90% CH_3OH and stirred for 30 min. After concentration, the resulting compound was dried overnight under high vacuum. A white solid was obtained in quantitative yield (80 mg). $R_f = 0.38$ (CH_2Cl_2/CH_3OH/H_2O, 7:3:0.25); $[\alpha]_D^{20} = +4.76$ ($c = 0.42$, $CHCl_3$); ^{1}H NMR ($CDCl_3$): $\delta = 5.21$ (bs, 1H), 4.33 (bm, 1H), 4.18 (bs, 1H), 3.90–3.70 (overlapping signals, 4H), 2.30–2.26 (m, 4H), 1.64–1.39 (m, 4H), 1.37–1.07 (m, 48H), 0.85 ppm (t, 6H, $J = 7.2$ Hz); ^{13}C NMR ($CDCl_3$): $\delta = 174.3$, 173.7, 71.0, 69.9, 63.0, 34.3, 34.1, 31.9, 29.7, 29.7, 29.5, 29.4, 29.3, 29.1, 29.1, 24.8 (d, $J = 6.8$ Hz), 22.7, 14.1 ppm; ^{31}P NMR ($CDCl_3$): $\delta = 24.3$ ppm (decoupled); LRMS (ESI) *m*/*z* 661.4 [*M*−1], 423 [$M-H_{31}C_{15}CO$], 255.3 [$H_{31}C_{15}CO_2$]; HRMS (MALDI) for $C_{36}H_{71}NaO_8P$, found: 685.4779, calcd: 685.4784.

(*R*)-1,2-Dioleoylpropane-3-methylphosphonate ester (10b). Application of the procedure described above for **10a** led to compound **10b** as a colorless oil in quantitative yield (80 mg). $R_f = 0.38$ (CH_2Cl_2/CH_3OH/H_2O, 7:3:0.25); $[\alpha]_D^{20} = +3.14$ ($c = 1.4$, $CHCl_3$); ^{1}H NMR ($CDCl_3$): $\delta = 5.36$–5.27 (m, 4H), 5.22 (bs, 1H), 4.40–4.30 (bm, 1H), 4.19 (bs, 1H), 3.94–3.62 (overlapping signals, 4H), 2.35–2.26 (m, 4H), 1.99–1.95 (m, 8H), 1.60–1.50 (m, 4H), 1.40–1.20 (m, 40H), 0.85 ppm (t, 6H, $J = 7.2$ Hz); ^{13}C NMR ($CDCl_3$): $\delta = 174.3$, 173.7, 129.9, 129.7, 71.0, 69.9, 63.1, 34.2, 34.1, 31.9, 29.7, 29.7, 29.5, 29.3, 29.2, 29.1, 29.1, 29.0, 27.2 (d, $J = 3.8$ Hz), 24.8, 24.7, 22.7, 14.1 ppm; ^{31}P NMR ($CDCl_3$): $\delta = 24.7$ ppm (decoupled); LRMS (ESI) *m*/*z* 713.4 [*M*−1], 449.3 [$M-H_{33}C_{17}CO$], 281.2 [$H_{31}C_{15}CO_2$]; HRMS (MALDI) for $C_{40}H_{75}NaO_8P$, found: 737.5092, calcd: 737.5097.

Diethyl 1-palmitoyl-2-(*R*)-hydroxypropane-3-methylphosphonate ester (11a). To a solution of **8** (68 mg, 0.28 mmol) in anhydrous CH_2Cl_2 (2 mL) was added palmitic acid (68 mg, 0.27 mmol) followed by the addition of EDC (64 mg, 0.34 mmol) and DMAP (20 mg, 0.17 mmol) as a mixture in CH_2Cl_2 (0.5 mL) at 0 °C. The reaction was stirred overnight at 0 °C under Ar atmosphere until TLC showed complete consumption of the starting material. The reaction was quenched by the addition of CH_2Cl_2, followed by washing with H_2O and brine, drying over Na_2SO_4, and concentration in vacuo. The resulting residue was purified by FC using CH_2Cl_2/CH_3OH 10:0.3 to produce the desired compound as a colorless oil in 68% yield (88 mg, 0.19 mmol). $R_f = 0.5$ (CH_2Cl_2/CH_3OH 95:5), $[\alpha]_D^{20} = -3.17$ ($c = 0.82$, $CHCl_3$); ^{1}H NMR ($CDCl_3$): $\delta = 4.18$–4.06 (m, 6H), 4.03–4.96 (m, 1H), 3.82 (d, 2H $J = 7.6$ Hz), 3.66 (dd, 1H, $J = 4.0$, 10.0 Hz), 3.59 (dd, 1H, $J = 6.4$, 10 Hz) 3.28 (d, 1H, $J = 4.8$ Hz), 2.29 (t, 2H, $J = 7.6$ Hz), 1.60–1.54 (m, 2H), 1.33–1.22 (overlapping signals, m, 30H), 0.84 ppm (t, 3H, $J = 6.8$ Hz); ^{13}C NMR ($CDCl_3$): $\delta = 173.8$, 74.8, 74.7, 68.7, 66.6, 64.9, 62.5 (m), 34.0, 31.9, 29.7, 29.6, 29.6, 29.4, 29.3, 29.2, 29.1, 24.9, 22.6, 16.4 (d, $J = 6.2$ Hz), 14.1 ppm; ^{31}P NMR ($CDCl_3$): $\delta = 22.4$ ppm (decoupled); LRMS (CI) *m*/*z* 481.4 ([*M*+1], 92), 435.4 ([$M+1-C_2H_5O$], 100); HRMS (CI) for $C_{24}H_{50}O_7P$, found: 481.3294, calcd: 481.3289.

Diethyl 1-oleoyl-2-(*R*)-hydroxypropane-3-methylphosphonate ester (11b). Application of the procedure described above for **11a** but with oleic acid led to compound **11b** as a colorless oil in 53% yield (55 mg, 0.16 mmol). $R_f = 0.5$ (CH_2Cl_2/CH_3OH 95:5), $[\alpha]_D^{20} = -2.41$ ($c = 1.12$, $CHCl_3$); ^{1}H NMR ($CDCl_3$): $\delta = 5.35$–5.27 (m, 2H), 4.18–4.06 (m, 6H), 4.02–3.98 (m, 1H), 3.86–3.80 (m,), 3.67 (dd, 1H, $J = 4.0$, 10.0 Hz), 3.59 (dd, 1H, $J = 6.4$, 10 Hz), 3.22 (d, 1H, $J = 4.8$ Hz), 2.30 (t, 2H, $J = 7.6$ Hz), 2.00–1.95 (m, 4H), 1.64–1.54 (m 2H), 1.33–1.23 (overlapping signals, m, 26H), 0.85 ppm (t, 3H, $J = 6.8$ Hz); ^{13}C NMR ($CDCl_3$): $\delta = 173.8$, 129.9, 129.7, 74.8, 74.7, 68.7, 66.6, 64.9, 62.6 (m), 34.1, 31.9, 29.7, 29.7, 29.5, 29.3, 29.1, 29.1, 27.2, 27.1, 24.8, 22.6, 16.5, 16.4, 14.1 ppm; ^{31}P NMR ($CDCl_3$): $\delta = 22.4$ ppm (decoupled); LRMS (CI) *m*/*z* 507.4 ([*M*+1], 100), 489.4 ([*M*+1−OH], 54); HRMS (CI) for $C_{26}H_{52}O_7P$, found: 507.3459, calcd: 507.3451.

1-Palmitoyl-2-(*R*)-hydroxypropyl-3-methylphosphonate ester (12a). To a solution of **11a** (30 mg, 0.063 mmol) in CH_3CN (1 mL), BSA (0.232 mL, 0.94 mmol) and TMSBr (0.083 mL, 0.63 mmol) were added under Ar atmosphere. The reaction mixture was stirred for 3.5 h at room temperature until TLC indicated complete consumption of the starting material. Solvents were removed under reduced pressure, and the residue was dried in vacuo for 2 h, dissolved in 90% CH_3OH for 45 min, and concentrated under vacuum. The crude product was dissolved in CH_2Cl_2, and a 1:1 mixture of brine and water was added. The aqueous phase was extracted three times with CH_2Cl_2, and the organic phases were combined, dried (Na_2SO_4), and concentrated to give pure **12a** in 73% yield (19.4 mg, 0.046 mmol) in the form of a waxy solid. $R_f = 0.33$ (CH_2Cl_2/CH_3OH/H_2O 7:3:0.5); ^{1}H NMR ([D$_6$]DMSO): $\delta = 4.01$ (dd, 1H, $J = 4.2$, 11.4 Hz), 3.91 (dd, 1H, $J = 6.4$, 11.2 Hz), 3.79 (p, 1H, $J = 5.2$ Hz), 3.52–3.45 (overlapping signals, 4H), 2.29 (t, 2H, $J = 7.2$ Hz), 1.56–1.44 (m, 2H), 1.24 (bs, 24H), 0.85 ppm (t, 3H, $J = 6.8$ Hz); ^{13}C NMR ($CDCl_3$): $\delta = 173.8$, 74.2, 67.9, 64.7, 34.0, 31.9, 29.7, 29.7, 29.6, 29.4, 29.4, 29.3, 24.8, 22.7, 14.1 ppm; ^{31}P NMR ([D$_6$]DMSO): $\delta = 17.28$ ppm (decoupled); LRMS (FAB) 423.4 ([*M*−1], 100); HRMS (MALDI) for $C_{20}H_{41}NaO_7P$, found: 447.2482, calcd: 447.2488.

1-Oleoyl-2-(*R*)-hydroxypropane-3-methylphosphonate ester (12b). Application of the procedure described above for **12a** provided compound **12b** as a colorless oil in 66% yield (20 mg, 0.039 mmol). $R_f = 0.33$ (CH_2Cl_2/CH_3OH/H_2O 7:3:0.5); $[\alpha]_D^{20} = -4.4$ ($c = 1.12$, THF/CH_3OH, 2:1); ^{1}H NMR ([D$_6$]DMSO): $\delta = 5.36$–5.28 (m, 2H), 4.01–3.97 (m, 1H), 3.93–3.88 (m, 1H), 3.79–3.76 (m, 1H), 2.50–3.41 (m, 4H), 2.30–2.27 (m, 2H), 2.02–1.92 (m, 4H), 1.62–1.52 (m, 2H), 1.40–0.80 (m, 20H), 8.35 ppm (t, 3H, $J = 6.8$ Hz); ^{13}C NMR ($CDCl_3$): $\delta = 173.7$, 129.9, 129.6, 74.1, 68.0, 64.7, 34.0, 31.9, 29.7, 29.5, 29.3, 29.2, 27.2, 24.8, 22.7, 14.1 ppm; ^{31}P NMR ([D$_6$]DMSO): $\delta = 16.5$ ppm (decoupled); LRMS (FAB) 281.3 ([$C_{18}H_{33}O_2$], 100), 449.2 ([*M*−1], 17.3); HRMS (MALDI) for $C_{22}H_{43}NaO_7P$, found: 473.2639, calcd: 473.2644.

High-throughput Ca^{2+} measurements. RH7777 cells stably expressing either LPA_1, LPA_2, or LPA_3, and CHO cells stably expressing LPA_4 were plated on poly-L-lysine (0.1 mg mL^{-1})-coated black-wall clear-bottom 96-well plates (Corning Life Sciences, Acton, MA, USA) at a density of 5×10^4 cells per well or 4×10^4 cells per well (for CHO cells) and cultured overnight. The culture medium (DMEM containing 10% FBS for RH7777 cells, Ham's F-12 containing 10% FBS for CHO cells) was then replaced with modified Krebs solution (120 mM NaCl, 5 mM KCl, 0.62 mM $MgSO_4$, 1.8 mM $CaCl_2$, 10 mM HEPES, 6 mM glucose, pH 7.4), and the cells were serum-starved for 6 h (no starvation was applied to the CHO cells). Cells were loaded with Fura-2 AM for 35 min (1 h for CHO cells) in modified Krebs medium containing 2% (*v*/*v*) pluronic acid. The cells were rinsed with Krebs buffer and monitored in a FLEX station II instrument (Molecular Devices, Sunnyvale, CA, USA) at excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm for 2 min after the addition of the compound. Each test was performed in quadruplicate, and the mean values ± standard deviation (SD) were calculated.

Reporter gene assay. CV-1 (African green monkey kidney cells) were plated on 96-well plates at a density of 8×10^3 cells per well

in DMEM supplemented with 10% FBS. The next day, the cells were transiently transfected with 125 ng of pGL3-PPRE-acyl-CoA oxidase luciferase, 62.5 ng of pcDNA3.1-PPARγ, and 12.5 ng of pSV-β-galactosidase (Promega) using LipofectAMINE 2000 (Invitrogen). 24 h after transfection, the cells were treated with 1% FBS-supplemented Opti-MEMI (Invitrogen) containing DMSO or test compound (10 μM) in DMSO for 20 h. Luciferase and β-galactosidase activities were measured with the Steady-Glo Luciferase Assay System (Promega) and the Galacto-Light Plus System (Applied Biosystems), respectively. Samples were run in quintuplicate, and the mean values ±SD were calculated. Data are representative of at least two independent transfections. Student's *t* test was used for null hypothesis testing, and $p < 0.01$ was considered significant.

Responses of cancer cell lines. The reverse phase protein array (RPPA) is a sensitive, high-throughput, quantitative assay for studying network signaling.[48–50,59,60] Cells were seeded in 96-well plates and serum-starved overnight prior to stimulation with 18:1 LPA or the indicated MP-PA or MP-LPA analogue at 0, 0.1, 1.0, or 10 μM for 5 min. For HT-29 cells, triplicate assays were performed. Cells were washed with PBS and lysed in RPPA lysis buffer [1% Triton X-100, 50 mM HEPES (pH 7.4), 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 100 mM NaF, 10 mM $NaPP_i$, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride] prior to denaturing cellular proteins with SDS and making serial dilutions. Samples were then transferred to 384-well plates and heated before printing the proteins onto nitrocellulose-coated slides using an automated robotic arrayer (Genomic Solutions, Inc., Ann Arbor, MI, USA). Slides were probed using the indicated antibodies along the *x*-axis of each figure; antibodies recognize either phosphorylated or total protein in pairs. Results were analyzed by MicroVigene (VigeneTech, Inc., North Billerica, MA, USA), a customized software for automatic spot finding, background subtraction, and curve fitting. Phosphorylation was quantified based on the spot density normalized to protein loading and presented as the ratio of phosphorylated protein to total protein along the *y*-axis. The use of ratios resolves any potential problems with protein loading.

RNA extraction and RT-PCR analysis. Total RNA was extracted from freshly harvested HT-29 cells using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and treated with amplification-grade DNase I to remove any contaminating genomic DNA. Reverse-transcription polymerase chain reaction (RT-PCR) was performed in a reaction mixture volume of 50 μL, using Superscript III One-step RT-PCR system (Invitrogen, Carlsbad, CA, USA) containing 25 μL 2× buffer, 2 μL Platinum Taq DNA polymerase, 400 ng total RNA, 200 nM each primer (LPA_{1-5} or β-actin). RT-PCR was carried out under the following conditions: incubation at 55 °C for 15 min followed by denaturation at 94 °C for 2 min, and then 30 cycles of amplification with denaturation at 94 °C for 15 s, annealing at 58 °C for 30 s, and extension at 68 °C for 1 min. RT-PCR products were separated on agarose gels and confirmed by size. The gene- and species-specific primers are shown below.

Gene	Forward primer	Reverse primer
LPA_1	5′-CCGCCGCTTCCATTTTCCTATTTA	CAGTTCCAGCCCACACTGGGTAT-3′
LPA_2	5′-GCCTACCTCTTCCTCATGTTCC	CATGAGCAGGAAGACAAGCAGG-3′
LPA_3	5′-CACATGTCAATCATGAGGATG	CCTAAGACGGTCATCACTGTCTTC-3′
LPA_4	5′-GAAGGCTTCTCCAAACGTGTCTG	TTCAGAGTTGCAAGGCACAAGG-3′
LPA_5	5′-CTGATGCTCATCAACGTGGACC	TAGGGCACGAAGCACAGCAG-3′
β-actin	5′-GACAACGGCTCCGGCATGTG	GGCTGGGGTGTTGAAGGTCTCAA-3′

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