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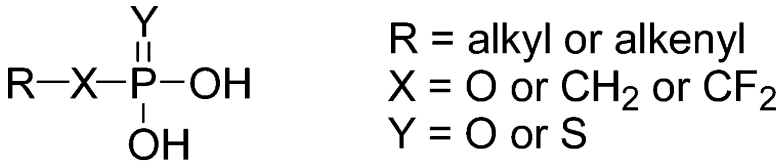

Modified fatty alcohol phosphates

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Synthesis, Structure−Activity Relationships, and Biological Evaluation of Fatty Alcohol Phosphates as Lysophosphatidic Acid Receptor Ligands, Activators of PPARγ, and Inhibitors of Autotaxin†

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We previously reported that fatty alcohol phosphates (FAP) represent a minimal pharmacophore required to interact with lysophosphatidic acid (LPA) receptors. To improve the activity of the first-generation saturated FAP series, a structure−activity relationship (SAR) study was carried out that includes modifications to the headgroup and alkyl side chain of the FAP pharmacophore. A series of unsaturated (C_{10}−C_{18}) FAP, headgroup-modified hydrolytically stable saturated (C_{10}−C_{18}) alkyl phosphonates, and saturated and unsaturated (C_{10}−C_{18}) thiophosphate analogues were synthesized and evaluated for activity in RH7777 cells transfected with individual LPA_{1-3} receptors, in PC-3 cells and in human platelets that endogenously express all three isoforms. In this series we identified several LPA_1- and LPA_3-selective antagonists with IC_{50} values in the nanomolar range. Oleoyl-thiophosphate (**15g**) was shown to be a pan-agonist, whereas tetradecyl-phosphonate (**16c**) was identified as a pan-antagonist. These compounds were also tested for the ability to activate the transcription factor PPARγ, an intracellular receptor for LPA, in CV1 cells transfected with the PPRE-Acox-Rluc reporter gene. All the FAP tested, along with the previously reported LPA GPCR antagonists dioctanoyl glycerol pyrophosphate (**2**), Ki16425 (**6**), and the agonist OMPT (**3**), were activators of PPARγ. The pan-agonist oleoyl-thiophosphate (**15g**) and pan-antagonist tetradecyl-phosphonate (**16c**) mimicked LPA in inhibiting autotaxin, a secreted lysophospholipase D that produces LPA in biological fluids.

Introduction

Lysophosphatidic acid (1-acyl-2-hydroxy-*sn*-glycero-3-phosphate, LPA, **1**; Figure 1) is a member of phospholipid growth factor (PLGF) family. LPA is generated by activated platelets and tumor cells and elicits a wide range of biological effects including the stimulation of cell proliferation, and migration, as well as the promotion of cell survival, platelet aggregation, apoptosis, and smooth muscle contraction.[1,2] LPA mediates many of these cellular responses through the activation of members of endothelial differentiation gene (EDG) family of G-protein-coupled receptors LPA_1/EDG-2, LPA_2/EDG-4, LPA_3/EDG-7.[3−9] Recently two non-EDG LPA receptors, LPA_4/GPR23 and the nuclear transcription factor/receptor PPARγ, have been reported.[10,11] LPA is a product of lysophospholipase D, also known as autotaxin, a phosphodiesterase that cleaves lysophosphatidylcholine to LPA and choline.[12,13]

With a growing understanding of the involvement of LPA in both normal physiology and pathology it is evident that LPA receptor agonists and antagonists may have therapeutic potential in treating various diseases. LPA mimics are of therapeutic interest in wound healing[14,15] and protection against radiation- and chemotherapy-induced apoptosis.[16] Alternatively, LPA antagonists may have therapeutic potential in the treatment of thrombosis elicited by plaque rupture,[17,18] ovarian cancer,[19] atherosclerosis,[20] ischemic reperfusion injury,[21] and inflammation.[22]

Understanding the physiological and pathological role of LPA is a challenge due to the complexity of LPA signaling. Most cells express multiple LPA plasma membrane receptors, which upon stimulation interact with multiple heterotrimeric G proteins. The recent discovery of PPARγ as an intracellular LPA receptor and the likely presence of more plasma membrane receptors for LPA add to the complexity of the system. To decipher the physiological role of individual receptors, and to take advantage of LPA analogues in treating various LPA-associated conditions, it is necessary to identify subtype-selective agonists and antagonists.

Despite their promising therapeutic potential, only a few agents with limited subtype selectivity have been reported to date. A number of attempts have been made

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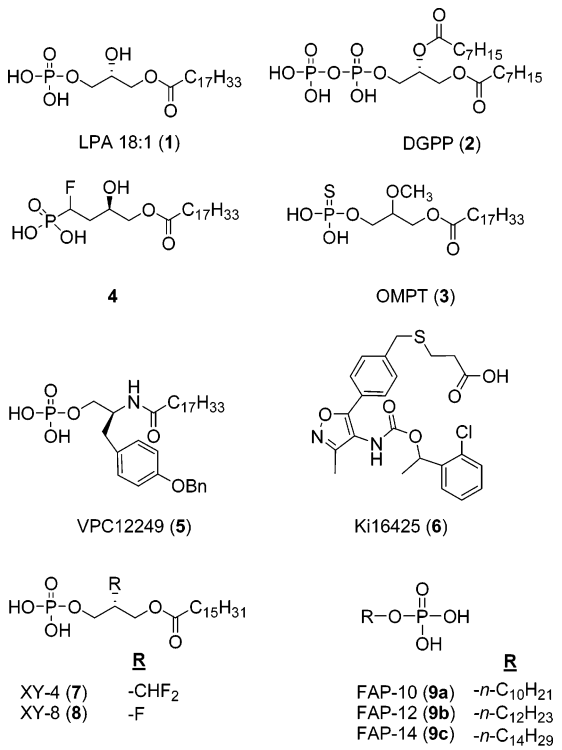
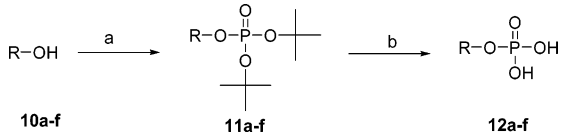


Figure 1. Chemical structures of LPA, saturated FAP, and other known LPA receptor ligands.

to identify LPA receptor agonists and antagonists.[1] *N*-Acylethanolamide phosphate (NAEPA) and *N*-palmitoylserine phosphate (Ser-PA) have been identified as LPA receptor agonists in mammalian cell lines.[23,24] Ser-PA has been documented to antagonize both LPA-induced platelet aggregation[25] and oocyte depolarization.[26] Fisher et al. showed that the short-chain phosphatidates, dioctanoyl glycerol pyrophosphate (DGPP, **2**) and dioctanoyl phosphatidic acid (PA 8:0), as selective antagonists of the LPA_1 and LPA_3, have 1 order of magnitude preference for LPA_3.[27] Rational modifications of the glycerol backbone and the headgroup of LPA resulted in the identification of OMPT (**3**),[28,29] a phosphothionate analogue, and a monofluoro phosphonate analogue **4** as selective LPA_3 receptor agonists.[30] VPC12249 (**5**) was characterized as a selective LPA_1 and LPA_3 antagonist, whereas a nonlipid compound Ki16425 (**6**) was shown to be an antagonist of $LPA_{1/3}$.[31,32] Two fluorinated acyl migration-resistant LPA analogues, XY-4 (**7**) and XY-8 (**8**), have been identified as selective PPARγ agonists.[11]

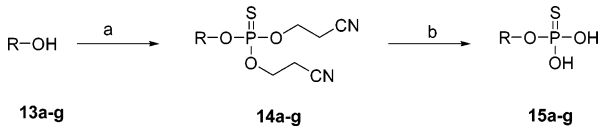
We previously reported a rational drug design driven identification of fatty alcohol phosphates (FAP), which lack a glycerol backbone and represent the minimal pharmacophore that interacts with LPA receptors, as subtype-selective LPA receptor agonists and antagonists.[33] n-Decyl- (**9a**, FAP-10), n-dodecyl- (**9b**, FAP-12), and n-tetradecyl- (**9c**, FAP-14) phosphates were identified as selective LPA_2 agonists and LPA_1/LPA_3 antagonists (Figure 1). In the current series of modifications we investigated the effect of headgroup modification, side chain modification, and double bonds at specific positions in the side chain on the ligand properties at LPA_{1-3} GPCR and PPARγ. Here we provide evidence that modification of the phosphate and/or hydrophobic chain has significant effects on the ability of FAP

Scheme 1[a]



[a] Reagents and conditions: (a) (i) di-*tert*-butyl *N,N*-diisopropylphosphoramidite, 1*H*-tetrazole, CH_2Cl_2; (ii) 50% H_2O_2, 68−82%; (b) TFA, CH_2Cl_2, 78−86%.

Scheme 2[a]



[a] Reagents and conditions: (a) (i) bis(2-cyanoethyl) *N,N*-diisopropylphosphoramidite, 1*H*-tetrazole, CH_2Cl_2, rt; (ii) sulfur, reflux, 72−84%; (b) (i) KOH, MeOH; (ii) dil HCl, 70−86%.

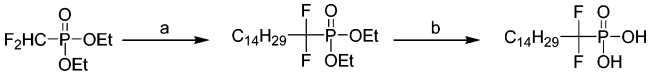
analogues to selectively activate or inhibit LPA_{1-3} receptors. The results include identification of several LPA_1- and LPA_3-selective antagonists with IC_{50} values in the nanomolar range. We also identified oleoyl-thiophosphate (**15g**) as a novel pan-agonist and tetradecyl-phosphonate (**16c**) as a pan-antagonist with respect to LPA GPCR. We also show that all the FAP we examined are activators of PPARγ and that the pan-agonist (**15g**) and pan-antagonist (**16c**) compounds mimic LPA in inhibiting autotaxin/lysophospholipase D.

Chemistry. The synthesis of alkenyl phosphates (**12a**−**f**), as outlined in Scheme 1, was carried out according to the method of Lindberg et al.,[34] with the exception that hydrogen peroxide was used for the oxidation step instead of *m*-chloroperbenzoic acid. Commercially available unsaturated fatty alcohols (**10a**−**f**) were treated with a mixture of 1*H*-tetrazole and di-*tert*-butyl *N,N*-diisopropylphosphoramidite in anhydrous methylene chloride followed by hydrogen peroxide oxidation to give di-*t*-Boc-protected fatty alcohol phosphates (**11a**−**f**). Boc- protected FAP were deprotected with TFA to yield the corresponding unsaturated FAP (**12a**−**f**).

The synthesis of thiophosphates (**15a**−**g**) is outlined in Scheme 2. The protected thiophosphoric acid *O,O′*-bis-(2-cyanoethyl) ester *O″*-alkyl/alkenyl esters were synthesized using a modified method of Haines et al.[35] Commercially available fatty alcohols (**13a**−**g**) were treated with a mixture of 1*H*-tetrazole and bis(2-cyanoethyl) *N,N*-diisopropylphosphoramidite in anhydrous methylene chloride followed by reflux in the presence of elemental sulfur to give bis-cyanoethyl-protected fatty alcohol thiophosphates (**14a**−**g**). These protected thiophosphates were treated with methanolic KOH, followed by acidification, to yield the required thiophosphates (**15a**−**g**). The n-alkyl phosphonate analogues (**16a**−**d**) used in this study were purchased from commercial sources.

The tetradecyl-difluorophosphonate analogue was synthesized (Scheme 3) in two steps using diethyl difluoromethanephosphonate (**17**) as the starting material.[36] Compound **17** was treated with LDA at −78 °C followed by reacting the anion with tetradecyl bromide to give the protected phosphonate **18**. Compound **18** was deprotected using bromotrimethylsilane to yield the required difluorophosphonate compound (**19**).

Scheme 3[a]



17 **18** **19**

[a] Reagents and conditions: (a) (i) LDA, −78 °C, THF; (ii) $C_{14}H_{29}Br$, 40%; (b) (i) TMSBr, CH_2Cl_2, 6 h, rt; (b) MeOH/H_2O, 78%.

Biology. Compounds were tested for their ability to induce or inhibit LPA-induced calcium transients in RH7777 rat hepatoma cells stably expressing LPA_1, LPA_2, and LPA_3 receptors and in PC-3 cells that express LPA_{1-3} endogenously using a FlexStation II automated fluorometer (Molecular Devices, Sunnyvale, CA).[27,33] The detailed protocol used is included in the Experimental Section. Selected compounds were tested for activation of human platelets[17,18] and PPARγ activation in CV1 cells, transfected with an acyl-coenzyme A oxidase-luciferase (PPRE-Acox-Rluc) reporter gene construct as previously reported.[37] The two compounds with pan-agonist and antagonist properties were assayed for the effect on lysophospholipase D activity.[38]

Results and Discussion

According to our original two-point contact model,[39,40] both a polar phosphate headgroup and a hydrophobic tail are required for specific interactions with LPA GPCR.[27] On the basis of this model, we identified **2** and PA 8:0 as selective LPA_1 and LPA_3 antagonists and FAP as subtype-selective agonists/antagonists of LPA_{1-3} receptors.[27,33] Bandoh et al. showed, using Sf-9 insect cells transiently transduced by LPA GPCR, that LPA_3 prefers unsaturated fatty acyl LPA species over saturated LPAs.[41] Replacement of the phosphate with a phosphonate renders compounds metabolically stable against degradation by lipid phosphate phosphatases. The phosphonate modification also affects ligand−receptor interactions by reducing charge density on the polar headgroup. Phosphonate analogues of LPA have been studied recently and are found to be less potent than LPA.[42,43] Alternatively, thiophosphate in place of phosphate yielded metabolically stable compounds with increased charge on the polar headgroup such as **3**, a selective LPA_3 agonist.[28,29]

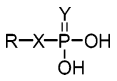
To explore the effects of these modifications along with variations in the side chain of FAP structure, we synthesized a series of FAP analogues with an unsaturation at different positions in the alkyl side chain (**12a**−**f**), thiophosphates (**15a**−**g**), and phosphonates (**16a**−**d**, **19**). These new analogues were evaluated as agonists and antagonists with respect to LPA_{1-3}. Saturated FAP analogues containing 10, 12, or 14 carbons (**9a**−**c**) were previously shown to be the most effective agonists and/or inhibitors at LPA_{1-3} in our initial study.[33] For this reason we synthesized and characterized modified FAP analogues with these optimum chain lengths.

Each FAP analogue was tested for the ability to induce Ca^{2+} transients in RH7777 cells transfected with LPA_{1-3} receptors (agonism), as well as the ability to inhibit LPA-induced Ca^{2+} transients in the same cells (antagonism) (Table 1). Every FAP analogue used in this study was tested for activity in nontransfected RH7777 cells. None of the FAP analogues, when applied up to a concentration of 30 μM, induced intracellular Ca^{2+} transients or inhibited the endogenous purinergic receptor response elicited by 10 μM ATP (data not shown). The effects of unsaturation at different positions, modification of headgroup to phosphonate, difluoro phosphonate, and thiophosphate with/without unsaturation on the activity of C-14 analogues at LPA_{1-3} receptors, are shown in Figure 2. These modifications dramatically changed the pharmacological properties of FAP on LPA_{1-3} receptors. The monounsaturated FAP analogues (**12a**−**e**) showed increased potency and/or efficacy when compared to saturated analogues, except C-10 analogues, without changing their properties as agonists or antagonists at the LPA_2 and LPA_3 receptors (Table 1). The position of the double bond also had an impact on the activity. Comparison of the activities between n-decenyl regioisomers **12a** and **12b** suggests that the C_9=C_{10} double bond, as found in LPA 18:1, was preferred over C_4=C_5 in activating the LPA_2 receptor (EC_{50} = 3800 nM for **12a** versus >10000 nM for **12b**). Though **12b** (K_i = 370 nM) was moderately more active than **12a** (K_i = 504 nM), the preference for the double bond position was much less pronounced for inhibition of the LPA_3 receptor. Similarly, the LPA_2 receptor showed preference for C_9=C_{10} unsaturation between the tetradecenyl isomers **12d** (EC_{50} = 397 nM) and **12e** (EC_{50} = 4100 nM), and LPA_3 showed no significant preference for double bond position. In contrast, LPA_1 preferred C_{11}=C_{12} over C_9=C_{10} between **12d** (K_i = 1146 nM) and **12e** (K_i = 457 nM), indicating the possibility of a differential conformational requirement in the side chain for each of the three LPA receptors (Figure 2). In the unsaturated series, only the tetradecenyl compounds (**12d**, **12e**) antagonized the LPA response at LPA_1 receptor. This further supports our hypothesis that the length of the side chain is critical for interaction with LPA receptors.

The replacement of phosphate with a thiophosphate as the headgroup in 10-, 12-, and 14-carbon saturated FAP analogues (**15a**−**c**) had a significant impact on their agonist/antagonist properties at all three LPA receptor subtypes. At LPA_1, the thiophosphate modification completely abolished the inhibitory effects of the original FAP analogues. At LPA_2 on the other hand, the thiophosphate invariably increased the efficacy of the original FAP to 100%. At the LPA_3 receptor, the saturated thiophosphate FAP analogues consistently showed improved inhibition of the LPA response compared to the original FAP. Dodecyl-thiophosphate (**15b**) is the most potent agonist and antagonist in the saturated thiophosphate analogues at LPA_2 (EC_{50} = 1000 nM) and LPA_3 (K_i = 14 nM), respectively. These results are consistent with our two-point contact model as the increase in the charge density, influenced by the properties of the hydrophobic tail, increased the agonist or antagonist properties of the FAP.

Next, we investigated the effect of combining a thiophosphate headgroup with monounsaturation (C_9=C_{10}) in the side chain. The combination of the thiophosphate headgroup with C_9=C_{10} unsaturation resulted in analogues (**15d**−**f**), with properties that were a combination of the properties of saturated thiophosphates and unsaturated FAP, substantially lowering the EC_{50} and IC_{50} values. Similar to the saturated thio analogues, compounds **15d**−**f** were inactive at the LPA_1 receptor.

Table 1. Effects of FAP Analogues **12a**−**f**, **15a**−**g**, **16a**−**d**, and **19** on LPA_{1-3} Transfected RH7777 Cells and Comparison of the Activities with the Previously Reported Compounds



				LPA1		LPA2		LPA3		PC-3	
compd	X	Y	R	EC_{50} (E_{max})[a] nM	IC_{50} (K_i) nM	EC_{50} (E_{max}) nM	IC_{50} (K_i) nM	EC_{50} (E_{max}) nM	IC_{50} (K_i) nM	EC_{50} (E_{max}) nM	IC_{50} (K_i) nM
9a[b]	O	O	$(CH_2)_9CH_3$	NE[c]	NE	1800 (82)	NE	NE	384 (121)	ND[d]	ND
9b[b]	O	O	$(CH_2)_{11}CH_3$	NE	2800 (1354)	3100 (50)	NE	NE	128 (61)	ND	ND
9c[b]	O	O	$(CH_2)_{13}CH_3$	NE	2300 (1082)	NE	NE	NE	422 (211)	ND	ND
12a	O	O	$(CH_2)_8CH{=}CH_2$	NE	>10000	3800 (100)	NE	NE	770 (504)	NA[e]	1510 (574)
12b	O	O	$(CH_2)_3CH{=}CH(CH_2)_4CH_3$	NE	>10000	>10000	NE	NE	830 (370)	NA	1300 (735)
12c	O	O	$(CH_2)_8CH{=}CHCH_2CH_3$	NE	>10000	717 (78)	NE	NE	32 (27)	NA	916 (390)
12d	O	O	$(CH_2)_8CH{=}CH(CH_2)_3CH_3$	NE	3000 (1146)	397 (58)	NE	NE	96 (58)	NA	241 (123)
12e	O	O	$(CH_2)_{10}CH{=}CHCH_2CH_3$	NE	2200 (457)	4100 (75)	NE	NE	103 (40)	ND	ND
12f	O	O	$(CH_2)_8CH{=}CH(CH_2)_7CH_3$	NE	NE	NE	NE	NE	NE	− (11)	NA
15a	O	S	$(CH_2)_9CH_3$	NE	NE	4570 (100)	NE	NE	122 (49)	NA	1220 (521)
15b	O	S	$(CH_2)_{11}CH_3$	NE	NE	1000 (100)	NE	NE	28 (14)	NA	2838 (1300)
15c	O	S	$(CH_2)_{13}CH_3$	NE	NE	2500 (100)	NE	NE	162 (76)	NE	NE
15d	O	S	$(CH_2)_8CH{=}CH_2$	NE	NE	>10000(56)	NE	NE	340 (128)	NA	1000 (533)
15e	O	S	$(CH_2)_8CH{=}CHCH_2CH_3$	NE	NE	677 (100)	NE	NE	27 (14)	− (27)	2972 (1460)
15f	O	S	$(CH_2)_8CH{=}CH(CH_2)_3CH_3$	NE	NE	480 (150)	NE	NE	28 (14)	− (40)	938 (397)
15g	O	S	$(CH_2)_8CH{=}CH(CH_2)_7CH_3$	193 (80)	NE	244 (175)	NE	546 (78)	NE	− (30)	NA
16a	CH_2	O	$(CH_2)_8CH_3$	NE	NE	NE	NE	NE	1200 (680)	NA	3122 (1500)
16b	CH_2	O	$(CH_2)_{10}CH_3$	NE	NE	NE	NE	NE	654 (303)	NA	2638 (1270)
16c	CH_2	O	$(CH_2)_{12}CH_3$	NE	~10000	NE	5500 (3550)	NE	3100 (1120)	NA	9674 (4620)
16d	CH_2	O	$(CH_2)_{16}CH_3$	NE	NE	NE	NE	NE	NE	NE	NE
19	CF_2	O	$(CH_2)_{13}CH_3$	NE	2500 (788)[f]	~10000 (40)	NE	NE	1513 (575)	ND	ND
2[g]				NE	5500 (4300)	NE	NE	NE	454 (202)	ND	ND
5[h]				NE	WA	NE	NE	NE	1186 (588)	NA	WA
6[i]				NE	762 (425)	NE	NE	NE	301 (148)	NA	3384 (1740)
Ser-PA				1850 (100)	NE	>10000	NE	1600 (100)	NE	− (42)	NA
Tyr-PA				NE	NE	~11000	NE	NE	5570 (2325)	− (25)	WA[j]

[a] E_{max} = maximal efficacy of the drug/maximal efficacy of LPA 18:1, expressed as the percentage. [b] Previously reported in Virag et al. (ref 33). [c] NE = no effect was shown at the highest concentration (30 μM) tested. [d] ND = not determined. [e] NA = not applicable. [f] Partial antagonist with 40% inhibition of the LPA response. [g] Reported K_i values of **2** are 106 nM and 6.6 μM at LPA_3 and LPA_1, respectively (ref 27). [h] Reported K_i values of **5** are 137 nM and 428 nM at LPA_1 and LPA_3, respectively (ref 31). [i] Reported K_i values of **6** are 250 nM, 360 nM, and 5.6 μM at LPA_1, LPA_3, and LPA_2, respectively (ref 32). [j] WA = weak antagonist.

The unsaturated C_{12} and C_{14} thiophosphates showed increased potency at LPA_2 with a minimal change in the potency at LPA_3 receptor as compared to their saturated forms. The tetradec-9-enyl thiophosphate (**15f**) compound showed a unique pharmacology. It retained the features of the saturated thio analogues at LPA_1, as it had no effect on the LPA-induced Ca^{2+} mobilization. On the other hand, **15f** was found to be the best agonist at LPA_2 (EC_{50} = 480 nM) and most potent antagonist at LPA_3 (K_i = 14 nM) among all C-10, -12, and -14 thiophosphate analogues tested (Figures 2B and 2D). These differences in the ligand properties of the thiophosphate analogues at the LPA receptor subtypes may provide a practical advantage in improving subtype-selective agonists and antagonists, as short-chain thiophosphates interact selectively with LPA_2 and LPA_3 receptors.

Oleoyl-phosphate (**12f**), an unsaturated FAP analogue of oleoyl-LPA, neither inhibited nor activated Ca^{2+} mobilization in cells expressing LPA_{1-3}. However, it potentiated LPA response at all three LPA receptors when the two compounds were coapplied (data not shown). This observation led us to the hypothesis that by increasing the charge density of the **12f** headgroup by replacing the phosphate with a thiophosphate, we may turn this analogue into an agonist. To test this hypothesis, we synthesized and evaluated the oleoyl-thiophosphate (**15g**) at LPA_{1-3} receptors. In agreement with our prediction, compound **15g** was a partial agonist at LPA_1, LPA_3, and a potent and full agonist at LPA_2 with the EC_{50} of 244 nM (E_{max} = 175% of LPA response), lower than that of oleoyl-LPA (EC_{50} = 300 nM). The dose−responses of **15g**, comparing its effects with LPA 18:1 at LPA_{1-3} receptors, are shown in Figure 3.

The phosphonate analogues (**16a**−**d**) were weaker inhibitors and agonists at the LPA receptors than their phosphate counterparts, consistent with data reported previously.[42] However, tetradecyl-phosphonate (**16c**) inhibited LPA-induced Ca^{2+} mobilization at all three receptor subtypes with IC_{50} values in the micromolar range, thus becoming, to our knowledge, the first pan-antagonist of the LPA receptors (Figure 2). Compound **16c** caused a rightward shift in the LPA dose−response curve and reduced the maximal response elicited by 10 μM LPA in LPA_1, LPA_2, and LPA_3 expressing RH7777 cells (Figure 4). Compound **16c** is the only known inhibitor of the LPA_2 receptor subtype apart from **6** that exerts only a modest and partial inhibition.[32] Compound **16c**, with a simpler structure and phosphonate headgroup, is presumably resistant to degradation by lipid

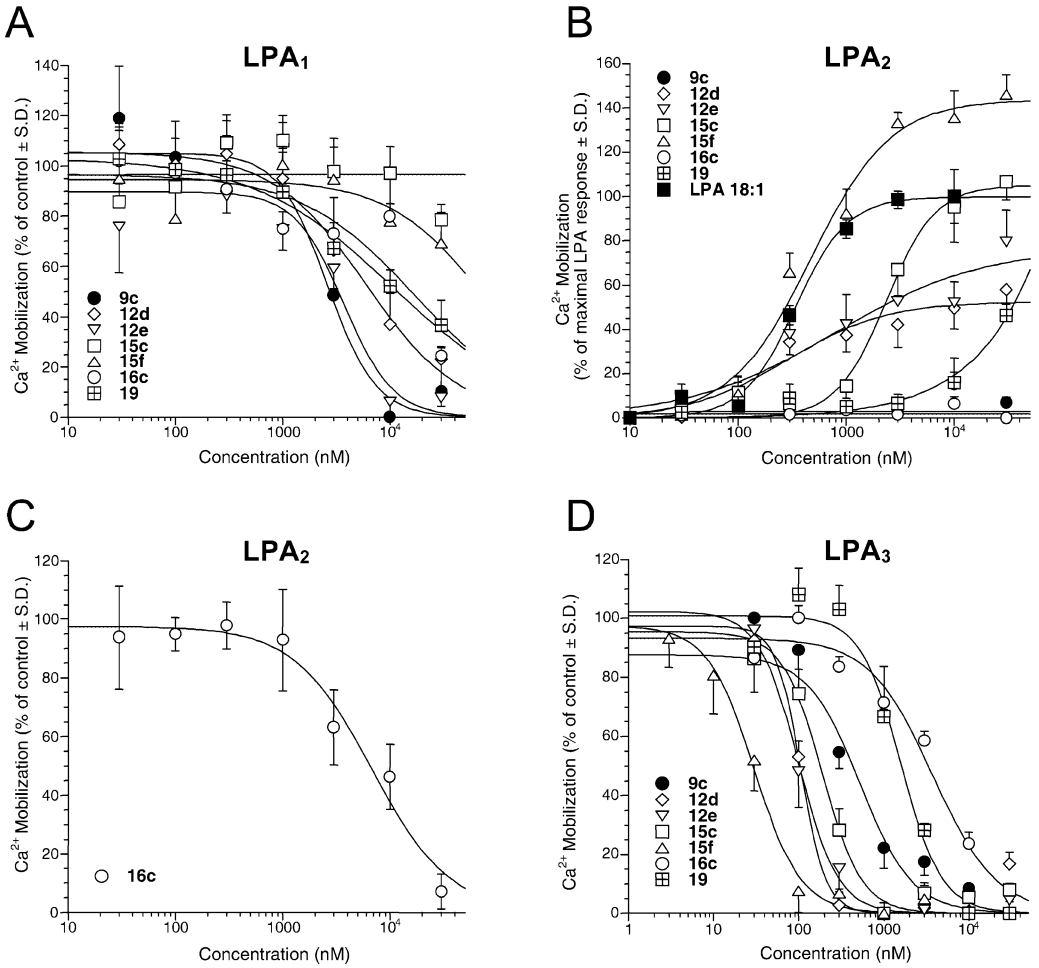


Figure 2. The effects of modified C-14 analogues on RH7777 cells stably transfected with the LPA_{1-3} receptors. 200 nM of LPA 18:1 was coapplied with increasing concentrations of C-14 analogues to RH7777 cells stably expressing LPA_1 and LPA_3. Increasing concentrations of various C-14 analogues were applied to measure their agonist properties at LPA_2. Data points represent an average of four measurements. (A) Inhibition of the LPA response by C-14 analogues at LPA_1; (B) activation of LPA_2 by C-14 FAP analogues; (C) inhibition of the LPA response by C-14 phosphonate **16c** at LPA_2; (D) inhibition of the LPA response by C-14 analogues at LPA_3.

phosphate phosphatases. These features make this molecule an attractive lead structure for further development of pan-antagonists for the LPA_{1-3} receptors. We synthesized compound **19**, which contains a difluorophosphonate group as an isosteric replacement for phosphonate in **16c**, and tested at LPA_{1-3} receptors. This compound retains the metabolic stability against phosphatases and at the same time increases the acidity of the phosphonate group, which presumably increases receptor binding. Increasing the acidity of phosphonate group by the addition of two fluorine atoms in compound **19** converted the compound from an antagonist to a weak and partial agonist with an EC_{50} of 10 μM (E_{max} = 40%) at the LPA_2 receptor. Compound **19** showed improved antagonist activity at LPA_3 compared to **16c**, while it showed partial antagonism at LPA_1.

All FAP antagonists have been tested for the reversibility of the antagonist effect. In these experiments RH7777 cells stably expressing LPA_2 or LPA_3 were exposed to LPA followed by LPA plus the inhibitor at 30 μM, and finally to LPA after a washout period. The LPA_1 receptor that shows very rapid and substantial desensitization could not be tested in this paradigm. However, as shown in Figure 5, **16c** exerted fully reversible antagonism in LPA_2 and LPA_3. Similar results were seen with the other FAP antagonists (data not shown). These observations are in complete agreement with our earlier report on FAP analogues.[33]

LPA was shown to activate mitogenic and motogenic signaling in PC-3 cells.[44] RT-PCR analysis of PC-3 cells, an androgen-independent human prostate cancer cell lines, showed expression of transcripts encoding all three LPA receptors.[45] We tested the FAP analogues in PC-3 cells, which unlike the transfected RH7777 cells endogenously express LPA_{1-3} receptors. Since PC-3 cells express LPA_{1-3} receptors, the effects shown by the FAP compounds (Table 1) represent the combination of the effects of these compounds at the three LPA receptors. These experiments confirmed the pharmacological properties of the FAP analogues obtained from RH7777 cells expressing each LPA receptor individually. Thiophosphate analogues (**15e** and **15f**) showed both independent activation and inhibition of LPA-induced Ca^{2+} transients in PC-3 cells, as they have different effects at each of the LPA_{1-3} receptors. Oleoyl-thiophosphate (**15g**) showed a maximal response of 30% of the maximal LPA response, with no inhibition of LPA response. These results are in consistent with the data obtained in transfected RH7777 cells. The consistency of the results obtained from PC-3 cells that endogenously express LPA receptors with those results obtained using transfected RH7777 cells validates our assay systems.

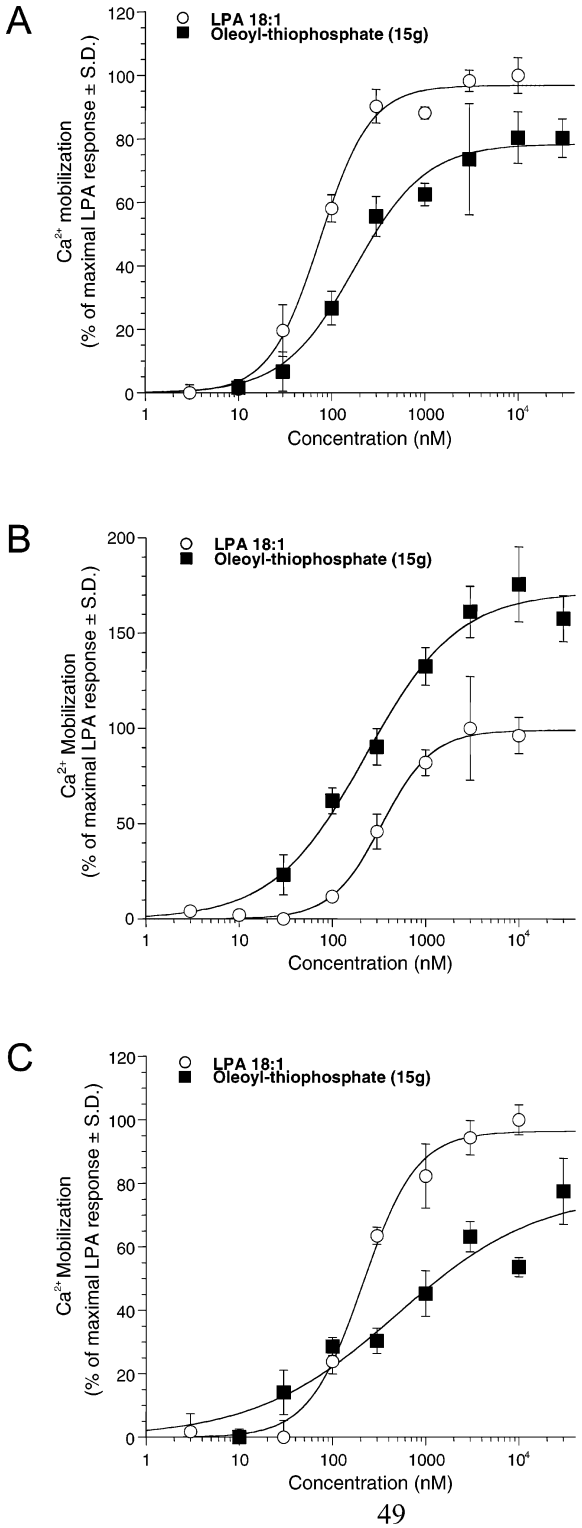


Figure 3. Oleoyl-thiophosphate (**15g**) is an agonist at LPA_1, LPA_2, and LPA_3 receptors expressed in RH7777 cells. Intracellular Ca^{2+} transients were measured in response to the application of increasing concentrations of **15g** and compared to transients elicited by the corresponding amount of LPA 18:1. Data points represent the average of four measurements. Dose−response relationships for LPA 18:1 and **15g** in RH7777 cells expressing LPA_1 (A), LPA_2 (B), and LPA_3 (C).

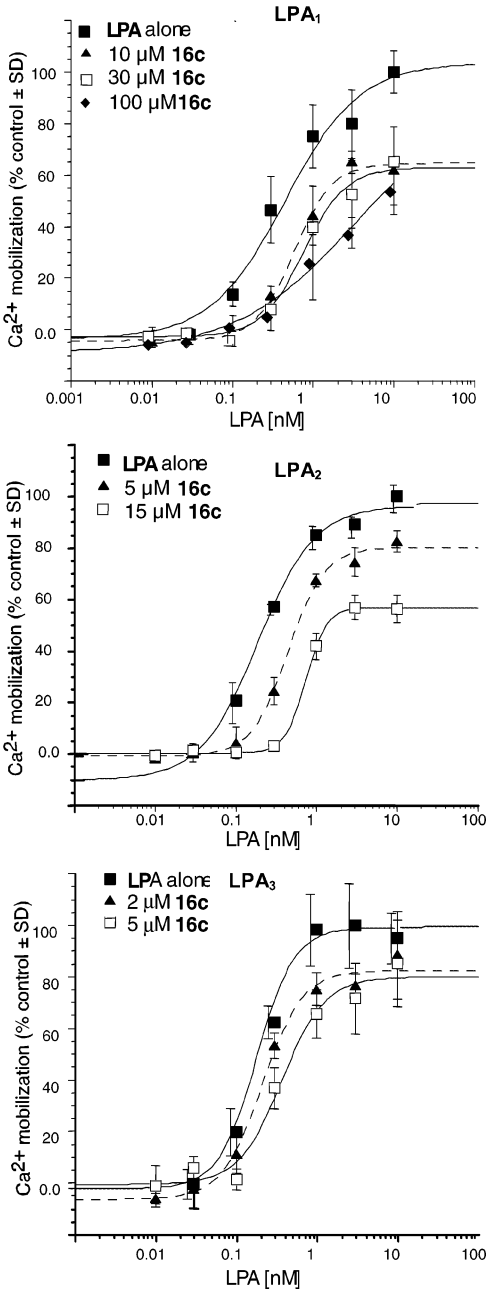


Figure 4. The effect of increasing concentrations of compound **16c** on the LPA dose−response curve in RH7777 cells stably transfected with LPA_{1-3} receptors. LPA-alone (closed squares) and LPA coapplied (closed trianges and open squares) with different concentrations of compound **16c**. Each point represents the mean ± s.d. for $n = 3$. Compound **16c** caused a rightward shift in the dose−response curve increasing the apparent EC_{50}. It also caused a modest inhibition of the maximal response to LPA.

LPA has been shown to activate platelets, and this effect is blocked by LPA GPCR antagonists.[17] LPA_1, LPA_2, and LPA_3 have been detected in platelets by RT-PCR. With the exception of the LPA receptor pan-antagonist **16c**, all compounds tested were agonists and elicited platelet shape change, which was reversible after 5 min (Table 2). The most potent compound was the pan-agonist **15g**, which induced shape change with an EC_{50} of 16 nM, only about 2-times higher than the EC_{50} of LPA 18:1. Compounds **12c** and **15e**, selective agonists of LPA_2, activated platelets with much lower potency, EC_{50} of 1.5 μM and 1.0 μM, respectively, than the pan-agonist **15g**. These results suggest the presence of functionally active LPA_2 receptors on platelets and also indicate that LPA_2 is not as important as LPA_1 and LPA_3 in platelet activation.

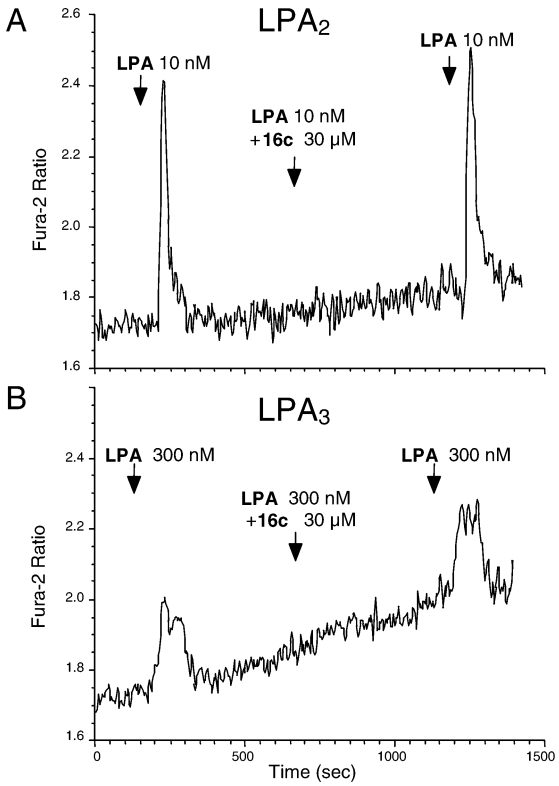


Figure 5. Reversible inhibition of the LPA_2 and LPA_3 response by compound **16c**. LPA_2 (A) or LPA_3 (B) cells were exposed to repeated concentrations of LPA or LPA plus 30 μM **16c**. Compound **16c** completely inhibited the response, which was fully recovered after a wash for both receptor types. The traces are representative of at least three experiments.

Table 2. Effect of Fatty Alcohol Phosphates and Fatty Alcohol Thiophosphates on Induction and Inhibition of Platelet Shape Change

compd	E_{max},[a] %	agonist activity, EC_{50} ±SD (μM)	antagonist activity, IC_{50} ±SD (μM)	n
9b	94 ± 3	0.25 ± 0.2	0.16 ± 0.05	3
9c	87 ± 9	0.43 ± 0.18	0.42 ± 0.28	5
			(0.3 ± 0.07)[b]	3
12c	89 ± 13	1.5 ± 0.8	0.7 ± 0.3	3
15e	73 ± 3	1.0 ± 0.02	0.7 ± 0.26	4
15g	112 ± 11	0.016 ± 0.005	0.018 ± 0.012	4
			(0.016 ± 0.01)[b]	3
16c	0	inactive	33 ± 8.0	3
LPA 16:0	120	0.010 ± 0.006	0.017 ± 0.010	3
LPA 18:1	120	0.009 ± 0.006	ND[c]	4

[a] E_{max} = maximal shape change induced by test compound/shape change induced by 0.025 μM LPA 16:0. Shape change induced by 0.025 μM LPA was 82 ± 6% (mean ± SD, n = 7) of maximal and set to 100%. Values are mean ± SD from different experiments with different platelet donors. Inhibitory activity was tested 5 min after addition of the test compounds, i.e., after the shape change induced by the compounds became reversible. [b] IC_{50} values after 30 min of incubation with the test compound. [c] ND = not determined.

All the compounds tested inhibited shape change induced by 25 nM LPA 16:0. The IC_{50} values of the fatty alcohol phosphates and fatty alcohol thiophosphates were in the range of the EC_{50} values. Since platelet LPA receptors show a homologous desensitization 5 min after activation,[46] it is possible that the fatty alcohol phosphates and fatty alcohol thiophosphates might exert their inhibition through desensitizing LPA receptors rather than receptor antagonism. To distinguish between these two possibilities, we reasoned that LPA receptor desensitization is reversible, whereas LPA receptor antagonism is not as long as the antagonist is present. Therefore, platelets were incubated for 30 min with LPA 16:0, the pan-agonist **15g** or the $LPA_{1/3}$ antagonist **9c** (which is an agonist in platelets). Indeed, after 30 min of incubation, platelets incubated with LPA 16:0 were again fully responsive to LPA, whereas the LPA response was inhibited in platelets incubated with **15g** or **9c**. The IC_{50} of **15g** and **9c** after 30 min incubation were 0.016 ± 0.01 μM and 0.3 ± 0.07 μM (mean ± SD, n = 3), respectively, which were similar to the IC_{50} values obtained after 5 min incubation (Table 2). There are two alternative explanations for these results: LPA receptors became desensitized or inhibited. Thus, the fatty alcohol phosphates and fatty alcohol thiophosphates might inhibit platelet LPA receptors through either receptor desensitization or antagonist action.

To compare the effects of these FAP analogues at LPA receptors with other available agonists and antagonists, we tested compounds **2**, **5**, **6**, Ser-PA, and *N*-acyl tyrosine phosphoric acid (Tyr-PA), in our RH7777 cell system. This comparison, where a single test system is used for all compounds, has the benefit of providing reliable information on the relative effectiveness of these compounds despite the inherent shortcomings the individual test systems may have. Our results were consistent with previously published data for **2**, **6**, and Ser-PA. However, we encountered differences for compounds **5** and Tyr-PA (Table 1). Compound **2** was identified by our group as a subtype-selective inhibitor for LPA_3 and LPA_1, with K_i values of 106 nM and 6.6 μM, respectively.[27] To test our high throughput screening system, we evaluated the effects of **2** in the same stably transfected RH7777 cell lines. The K_i values were 202 nM for LPA_3 and 4.3 μM for LPA_1 (Table 1). These results convincingly showed the reproducibility of the results of **2**, even after the modification of the original assay method. Compound **6** was synthesized and identified as a subtype-selective antagonist for LPA_1 and LPA_3 with a very weak inhibitory effect on LPA_2 with K_i values 250 nM, 360 nM, and 5.6 μM, respectively, using GTPγS loading assay in HEK293T cells transfected with LPA receptors.[32] When this compound was tested in our intracellular Ca^{2+} monitoring system, we obtained similar K_i values for LPA_1 (425 nM) and LPA_3 (148 nM); however, **6** seemed to inhibit LPA_3 better compared to LPA_1 (Table 1). Ser-PA and Tyr-PA were originally identified as inhibitors of LPA-induced platelet aggregation[25] and inhibitors of the LPA-induced Cl^- current in *Xenopus* oocytes.[26] In a mammalian cell line, however, Ser-PA was found to be an LPA-like agonist.[24] It was also shown to be an agonist at LPA_1 and LPA_2 when these receptor subtypes were heterologously expressed in TAg-Jurkat T-cells.[4] In our experiments, Ser-PA was a full agonist at LPA_1 (EC_{50} = 1.85 μM), but only a weak agonist at LPA_2. At LPA_3, Ser-PA was also a weak but full agonist with an EC_{50} value of 1.6 μM (Table 1).

An et al. showed that Tyr-PA did not affect LPA signaling at LPA_1 and LPA_2 receptors when applied at a concentration of 1 μM.[4] Tyr-PA in our experiments had no effect on LPA_1; however, it was found to be a weak agonist at LPA_2 (EC_{50} = 11 μM) and an inhibitor at LPA_3 (K_i = 2.3 μM) as shown in Table 1. Compound

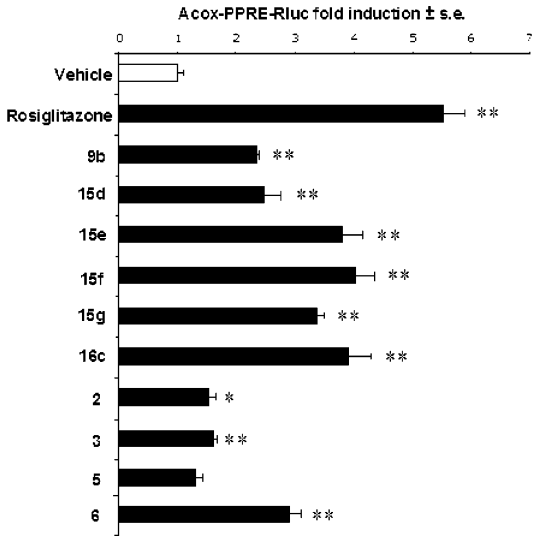


Figure 6. Results of in vitro PPARγ activation by selected compounds in CV1 cells transfected with PPARγ and PPRE-Acox-Rluc reporter gene, comparing the effects with rosiglitazone, a known PPARγ agonist. CV1 cells were treated with 1% DMSO or 10 μM of test compound dissolved in DMSO for 20 h. Luciferase and β-galactosidase activities (mean ± SEM) were measured in the cell lysate ($n = 4$). *$P < 0.05$ and **$P < 0.01$, significant differences over vehicle control.

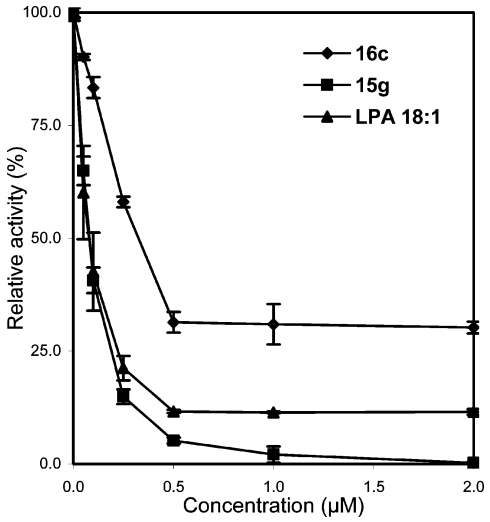


Figure 7. Concentration-dependent inhibition of autotaxin (ATX) phosphodiesterase activity by compounds **15g** and **16c**, as compared to LPA18:1. ATX activity was measured by the hydrolysis of the fluorescent substrate CPF4 (2 μM), as described in ref 38.

5 is a 2-substituted analogue of the NAEPA that was identified as a subtype-selective inhibitor of the LPA_1 and LPA_3 receptors, using a GTPγS-loading assay with cell membranes isolated from LPA_1-, LPA_2-, or LPA_3-expressing HEK293T cells. Compound **5** was a better antagonist at LPA_1 (K_i = 137 nM) than at LPA_3 (K_i = 428 nM).[31] In our experiments however **5** was only a weak inhibitor at LPA_1 and a better inhibitor at LPA_3 with a K_i value of 588 nM (Table 1). This value is in reasonable agreement with the published data in addition to the observation that **5** did not affect LPA signaling through LPA_2 (Table 1). Analogous to the FAP, these compounds also showed effects that are combination of effects at three LPA receptors on PC-3 cells, further validating our assay system.

In addition to its plasma membrane receptors, LPA was shown to be an agonist of the nuclear transcription factor PPARγ.[11] Many agents have been reported to activate PPARγ, including the thiazolidinedione family represented by rosiglitazone, oxidized phospholipids, fatty acids, eicosanoids, and oxidized LDL. Zhang et al. showed that unsaturated and alkyl ether analogues of LPA, 1,1-difluorodeoxy-(2*R*)-palmitoyl-*sn*-glycero-3-phosphate (**7**), its monofluoro analogue **8**, and the oxidized phosphatidylcholine 1-*O*-hexadecyl-2-azeleoyl-phosphatidylcholine, induced neointima formation, an early step leading to the development of atherogenic plaques, through PPARγ activation.[37] The SAR of neointima formation by LPA analogues in vivo was identical to PPARγ activation in vitro and different from LPA G-protein-coupled receptors.[37] We tested selected compounds including FAP-12 (**9b**), unsaturated thiophosphate analogues (**15d**−**g**), tetradecyl-phosphonate **16c**, previously reported LPA_1/LPA_3 antagonists **2**, **5**, **6**, and the selective LPA_3 agonist **3**, for PPARγ activation in vitro in CV1 cells using the PPRE-Acox-Rluc reporter gene assay. Interestingly, results from this assay (Figure 6) indicate that along with previously reported agonists (**3**) and antagonists (**2**, **5**, and **6**), FAP analogues, regardless of their LPA_{1-3} agonist/antagonist activities, can activate PPRE-Acox-Rluc reporter. These results are consistent with previously reported results,[37] in that LPA GPCR ligands can activate PPARγ. However, the results also emphasize that the SAR of PPARγ activation is different from GPCR.

Recent evidence shows that autotaxin, the major LPA-producing exo-phosphodiesterase/lysophospholipase D, exhibits product inhibition by LPA.[38] For this reason, we tested the pan-agonist **15g** and the pan-antagonist **16c** for their effects on autotaxin phosphodiesterase activity using a fluorescence-based biosensor.[38] The results showed that both analogues inhibited the enzyme, although **15g** was more effective than **16c** and even slightly more potent than LPA 18:1 itself (Figure 7). Compound **16c** exerted only a partial inhibition whereas **15g** showed a complete dose-dependent autotaxin inhibition at micromolar concentrations.

Conclusions

The present study extended our previously described two-point contact model as the minimal requirement to elicit specific interactions with LPA GPCR and provides further refinement of the minimal pharmacophore FAP by identifying modifications that allowed the synthesis of a pan-agonist and a pan-antagonist and several subtype-selective ligands. A systematic SAR study of the FAP pharmacophore with phosphonate, thiophosphate, and introduction of unsaturation in the side chain outlined important principles for the design of subtype-selective LPA receptor agonists and antagonists. The results of the FAP analogues, and previously reported LPA agonists and antagonists by other groups, obtained in transfected RH7777 cells expressing individual LPA receptors were consistent with results obtained in PC-3 cells that endogenously express LPA_{1-3} receptors. In addition to their ligand properties on LPA GPCR, we showed that FAP also activate nuclear transcription factor PPARγ with an SAR distinct from LPA GPCR. Based on the principles that emerged from the SAR of

9b, oleoyl-thiophosphate (**15g**) was synthesized and identified as a novel pan-agonist at all three LPA receptors. Tetradecyl-phosphonate (**16c**) was identified as a metabolically stable pan-antagonist that could serve as a lead structure for further development of LPA_{1-3} receptor antagonists that are stable against degradation by lipid phosphate phosphatases. Our results provide the first comprehensive evaluation of LPA GPCR ligands as activators of PPARγ. It was an unexpected surprise that all analogues tested, with the exception of **5**, were PPARγ activators, regardless of whether they were agonists or antagonists of LPA GPCR. Therefore, future synthesis must focus on developing LPA GPCR ligands that lack PPARγ agonist properties. Finally, two of our most interesting lead compounds, regardless of their agonist or antagonist properties on LPA GPCR and PPARγ, inhibited autotaxin/lysophospholipase D. Thus, the FAP analogues we have synthesized have multiple biological targets and serve as leads for future structural modifications to develop receptor type-, subtype-, and autotaxin-selective agents.

Experimental Section

General Methods. All reagents were purchased from Sigma-Aldrich Chemical Co., Fisher Scientific (Pittsburgh, PA), Bedukian Research (Danbury, CT), and Toronto Research Chemicals (North York, ON, Canada) and were used without further purification. Phosphonate analogues were purchased from Lancaster (Pelham, NH; n-decyl-phosphonate (**16a**)), PolyCarbon (Devens, MA; n-dodecyl-phosphonate (**16b**)), Alfa Aesar (Ward Hill, MA; n-tetradecyl-phosphonate (**16c**), and n-octadecyl-phosphonate (**16d**)). LPA 18:1, **2**, Ser-PA, and Tyr-PA were obtained from Avanti Polar Lipids (Alabaster, AL). Melting points were determined on a Thomas-Hoover capillary melting point apparatus and are uncorrected. Routine thin-layer chromatography (TLC) was performed on 250 μm glass-backed Uniplates (Analtech, Newark, DE). Flash chromatography was performed on pre-packed silica gel columns using a Horizon HPFC system (Biotage, Charlottesville, VA). ^{1}H and ^{31}P NMR spectra were obtained on a Bruker AX 300 (Billerica, MA) spectrometer. Chemical shifts for ^{1}H NMR are reported as parts per million (ppm) relative to TMS. Chemical shifts for ^{31}P NMR are reported as parts per million (ppm) relative to 0.0485 M triphenyl phosphate in $CDCl_3$. Mass spectral data was collected on a Bruker ESQUIRE electrospray/ion trap instrument in the positive and negative ion modes. Elemental analyses were performed by Atlantic Microlab Inc., Norcross, GA.

General Procedure for Synthesis Phosphoric Acid Di-*tert*-butyl Ester Alkenyl Esters (11a−f). To a stirred solution of alcohol (**10a**−**f**, 2.5 mmol) and di-*tert*-butyl *N,N*-diisopropylphosphoramidite (1.51 g, 4 mmol) in anhydrous methylene chloride (60 mL) was added 1*H*-tetrazole (578 mg, 8.25 mmol). After 30 min of stirring the mixture was cooled to 0 °C and 0.3 mL of 50% hydrogen peroxide was added. The mixture was stirred for 1 h, diluted with methylene chloride (100 mL), and washed with 10% sodium metabisulfite (2 × 50 mL), saturated sodium bicarbonate (2 × 50 mL), water (50 mL), and brine (50 mL). The organic layer was dried over anhydrous sodium sulfate, filtered, and concentrated under vacuum. The resulting crude products were purified by silica gel chromatography using hexane/ethyl acetate (7:3) to elute the desired products (**11a**−**f**).

Phosphoric acid di-*tert*-butyl ester dec-9-enyl ester (11a): Isolated as clear oil (75% yield). ^{1}H NMR ($CDCl_3$): δ 5.73−5.85 (m, 1H), 4.90−5.02 (m, 2H), 3.95 (q, J = 7.5 Hz, 2H), 2.03 (q, J = 7.1 Hz, 2H), 1.60−1.69 (m, 2H), 1.48 (s, 18H), 1.30 (br s, 10H); ^{31}P NMR ($CDCl_3$): δ 7.90 (s); MS: [M + ^{23}Na] at *m/z* 371.3.

Phosphoric acid di-*tert*-butyl ester dec-4-enyl ester (11b): Isolated as clear oil (68% yield). ^{1}H NMR ($CDCl_3$): δ 5.15−5.30 (m, 2H), 3.84 (q, J = 6.8 Hz, 2H), 2.05 (q, J = 7.0 Hz, 2H), 1.98 (q, J = 6.8 Hz, 2H), 1.58−1.62 (m, 2H), 1.42 (s, 18H), 1.22 (br s, 6H), 0.80 (t, J = 7.2 Hz, 3H); ^{31}P NMR (CD_3OD): δ 7.90 (s); MS: [M + ^{23}Na] at *m/z* 371.3.

Phosphoric acid di-*tert*-butyl ester dodec-9-enyl ester (11c): Isolated as clear oil (70% yield). ^{1}H NMR ($CDCl_3$): δ 5.15−5.45 (m, 2H), 3.88 (q, J = 6.6 Hz, 2H), 1.92−1.99 (m, 4H), 1.57−1.61 (m, 2H), 1.42 (s, 18H), 1.24 (br s, 10H), 0.89 (t, J = 7.5 Hz, 3H); ^{31}P NMR ($CDCl_3$): δ 7.80 (s); MS: [M + ^{23}Na] at *m/z* 399.5.

Phosphoric acid di-*tert*-butyl ester tetradec-9-enyl ester (11d): Isolated as clear oil (68% yield). ^{1}H NMR ($CDCl_3$): δ 5.34 (t, J = 5.2 Hz, 2H), 3.94 (q, J = 6.6 Hz, 2H), 2.01 (m, 4H), 1.60−1.69(m, 2H), 1.48 (s, 18H), 1.30 (br s, 18H), 0.90 (t, J = 7.4 Hz, 3H); ^{31}P NMR ($CDCl_3$): δ 7.90 (s); MS: [M + ^{23}Na] at *m/z* 427.4.

Phosphoric acid di-*tert*-butyl ester tetradec-11-enyl ester (11e): Isolated as clear oil (82% yield). ^{1}H NMR ($CDCl_3$): δ 5.28−5.40 (m, 2H), 3.94 (q, J = 6.5 Hz, 2H), 1.98−2.06 (m, 4H), 1.62−1.70 (m, 2H), 1.48 (s, 18H), 1.23 (br s, 14H), 0.95 (t, J = 7.4 Hz, 3H); ^{31}P NMR ($CDCl_3$): δ 8.10 (s); MS: [M + ^{23}Na] at *m/z* 427.4.

Phosphoric acid di-*tert*-butyl ester octadec-9-enyl ester (11f): Isolated as clear oil (72% yield). ^{1}H NMR ($CDCl_3$): δ 5.28−5.44 (m, 2H), 3.94 (q, J = 6.9 Hz, 2H), 1.90−2.10 (m, 4H), 1.60−1.70 (m, 2H), 1.48 (s, 18H), 1.28 (br s, 22H), 0.88 (t, J = 6.6 Hz, 3H); ^{31}P NMR ($CDCl_3$): δ 8.10 (s); MS: [M + ^{23}Na] at *m/z* 483.5.

General Procedure for Synthesis of Phosphoric Acid Monoalkenyl Esters (12a−f). To a solution of 100 mg of **11a**−**f** in anhydrous methylene chloride (20 mL) was added trifluroacetic acid (0.3 mL). The mixture was allowed to stir for 4 h, and TLC showed the completion of the reaction. Solvents were evaporated, and the residue was washed with methylene chloride (2 × 20 mL) and concentrated under vacuum to yield the desired phosphoric acid monoalkenyl esters (**12a**−**f**) as colorless oils.

Phosphoric acid monodec-9-enyl ester (12a): Isolated as an oil (85%). ^{1}H NMR (CD_3OD): δ 5.52−5.80 (m, 1H), 4.80−4.95 (m, 2H), 3.90 (q, J = 6.6 Hz, 2H), 2.01 (q, J = 6.9 Hz, 2H), 1.53−1.70 (m, 2H), 1.28 (br s, 10H); ^{31}P NMR (CD_3OD): δ 17.84 (s); MS: $[M - H]^-$ at *m/z* 235.2. Anal. ($C_{10}H_{21}O_4P\cdot 0.1H_2O$) C, H.

Phosphoric acid monodec-4-enyl ester (12b): Isolated as an oil (78%). ^{1}H NMR (CD_3OD): δ 5.25.35 (m, 2H), 3.84 (q, J = 6.8 Hz, 2H), 2.05 (q, J = 7.0 Hz, 2H), 1.98 (q, J = 6.8 Hz, 2H), 1.58−1.65 (m, 2H), 1.22 (br s, 6H), 0.80 (t, J = 7.2 Hz, 3H); ^{31}P NMR (CD_3OD): δ 17.45 (s); MS: $[M - H]^-$ at *m/z* 235.2. Anal. ($C_{10}H_{21}O_4P\cdot 0.5H_2O$) C, H.

Phosphoric acid monododec-9-enyl ester (12c): Isolated as an oil (82%). ^{1}H NMR (DMSO-d_6/CD_3OD): δ 5.20−5.40 (m, 2H), 3.82 (q, J = 6.6 Hz, 2H), 1.88−2.10 (m, 4H), 1.50−1.62 (m, 2H), 1.25 (br s, 10H), 0.88 (t, J = 7.2 Hz, 3H); ^{31}P NMR (CD_3OD): δ 16.22 (s); MS: $[M - H]^-$ at *m/z* 263.0. Anal. ($C_{12}H_{25}O_4P\cdot 0.6H_2O$) C, H.

Phosphoric acid monotetradec-9-enyl ester (12d): Isolated as an oil (84%). ^{1}H NMR ($CDCl_3$/CD_3OD): δ 5.12−5.30 (m, 2H), 3.84 (q, J = 6.5 Hz, 2H), 1.81−2.10 (m, 4H), 1.48−1.60 (m, 2H), 1.20 (br s, 14H), 0.78 (m, 3H); ^{31}P NMR (CD_3OD): δ 16.20 (s); MS: $[M - H]^-$ at *m/z* 291.4. Anal. ($C_{14}H_{29}O_4P\cdot 0.25H_2O$) C, H.

Phosphoric acid monotetradec-11-enyl ester (12e): Isolated as an oil (78%). ^{1}H NMR (CD_3OD): δ 5.18−5.30 (m, 2H), 3.88 (q, J = 6.6 Hz, 2H), 1.90−2.00 (m, 4H), 1.58−1.62 (m, 2H), 1.25 (br s, 14H), 0.86 (t, J = 7.1 Hz, 3H); ^{31}P NMR (CD_3OD): δ 16.20 (s); MS: $[M - H]^-$ at *m/z* 291.3. Anal. ($C_{14}H_{29}O_4P$) C, H.

Phosphoric acid monooctadec-9-enyl ester (12f): Isolated as an oil (86%). ^{1}H NMR (CD_3OD): δ 5.25−5.40 (m, 2H), 3.91 (q, J = 6.6 Hz, 2H), 1.90−2.05 (m, 4H), 1.55−1.70 (m, 2H), 1.26 (br s, 22H), 0.86 (t, J = 6.0 Hz, 3H); ^{31}P NMR (CD_3-

OD): δ 16.21 (s); MS: [M − H]⁻ at *m/z* 347.4. Anal. ($C_{18}H_{37}O_4P$·0.4H_2O) C, H.

General Method for Synthesis of Thiophosphoric Acid *O,O′*-Bis-(2-cyanoethyl) Ester *O″*-Alkyl/Alkenyl Esters (14a−g). A solution of alcohol (**13a−g**, 2.0 mmol), bis-(2-cyanoethyl) *N,N*-diisopropylphosphoramidite (1.085 g, 4 mmol) and 1*H*-tetrazole (420 mg, 6 mmol) in anhydrous methylene chloride (40 mL) was stirred for 30 min at room temperature, followed by the addition of elemental sulfur (200 mg), and the mixture was refluxed for 2 h. The reaction mixture was cooled to room temperature, and solvents were evaporated under vacuum. Addition of ethyl acetate (30 mL) precipitated excess sulfur, which was filtered out, and the solvent was evaporated to give the crude mixture. The mixture was purified by flash chromatography using hexanes/ethyl acetate (8:1) to give the desired products (**14a−g**) as colorless oils.

Thiophosphoric acid *O,O′*-bis-(2-cyanoethyl) ester *O″*-decyl ester (14a): Isolated as colorless oil (72% yield). ^{1}H NMR ($CDCl_3$): δ 4.21−4.35 (m, 4H), 4.10−4.13 (m, 2H), 2.8 (t, *J* = 6.3 Hz, 4H), 1.62−1.78 (m, 2H), 1.26 (br s, 14H), 0.88 (t, *J* = 6.0 Hz, 3H); ^{31}P NMR ($CDCl_3$): δ 84.56 (s); MS: [M + ^{23}Na] at *m/z* 383.4.

Thiophosphoric acid *O,O′*-bis-(2-cyanoethyl) ester *O″*-dodecyl ester (14b): Isolated as colorless oil (84% yield). ^{1}H NMR ($CDCl_3$): δ 4.26−4.33 (m, 4H), 4.07−4.19 (m, 2H), 2.8 (t, *J* = 6.2 Hz, 4H), 1.65−1.80 (m, 2H), 1.26 (br s, 14H), 0.88 (t, *J* = 6.6 Hz, 3H); ^{31}P NMR ($CDCl_3$): δ 84.55 (s); MS: [M + ^{23}Na] at *m/z* 411.4.

Thiophosphoric acid *O,O′*-bis-(2-cyanoethyl) ester *O″*-tetradecyl ester (14c): Isolated as clear oil (82% yield). ^{1}H NMR ($CDCl_3$): δ 4.25−4.33 (m, 4H), 4.08−4.17 (m, 2H), 2.8 (t, *J* = 6.0 Hz, 4H), 1.65−1.80 (m, 2H), 1.26 (br s, 18H), 0.88 (t, *J* = 6.6 Hz, 3H); ^{31}P NMR ($CDCl_3$): δ 84.56 (s); MS: [M + ^{23}Na] at *m/z* 439.5.

Thiophosphoric acid *O,O′*-bis-(2-cyanoethyl) ester *O″*-dec-9-enyl ester (14d): Isolated as clear oil (76% yield). ^{1}H NMR ($CDCl_3$): δ 5.72−5.91 (m, 1H), 4.88−5.05 (m, 2H), 4.22−4.32 (m, 4H), 4.07−4.17 (m, 2H), 2.8 (t, *J* = 6.3 Hz, 4H), 2.01 (t, *J* = 6.6 Hz, 4H), 1.65−1.78 (m, 2H), 1.31 (br s, 10H); ^{31}P NMR ($CDCl_3$): δ 84.58 (s); MS: [M + ^{23}Na] at *m/z* 381.3.

Thiophosphoric acid *O,O′*-bis-(2-cyanoethyl) ester *O″*-dodec-9-enyl ester (14e): Isolated as clear oil (80% yield). ^{1}H NMR ($CDCl_3$): δ 5.26−5.46 (m, 2H), 4.25−4.33 (m, 4H), 4.08−4.17 (m, 2H), 2.8 (t, *J* = 6.0 Hz, 4H), 2.07 (m, 2H), 1.62−1.76 (m, 2H), 1.31 (br s, 10H), 0.96 (t, *J* = 7.5 Hz, 3H); ^{31}P NMR ($CDCl_3$): δ 84.55 (s); MS: [M + ^{23}Na] at *m/z* 409.5.

Thiophosphoric acid *O,O′*-bis-(2-cyanoethyl) ester *O″*-tetradec-9-enyl ester (14f): Isolated as clear oil (75% yield). ^{1}H NMR ($CDCl_3$): δ 5.33−5.40 (m, 2H), 4.25−4.33 (m, 4H), 4.08−4.18 (m, 2H), 2.78 (t, *J* = 6.0 Hz, 4H), 2.02 (m, 2H), 1.64−1.80 (m, 2H), 1.31 (br s, 14H), 0.90 (t, *J* = 7.2 Hz, 3H); ^{31}P NMR ($CDCl_3$): δ 84.57 (s); MS: [M + ^{23}Na] at *m/z* 437.5.

Thiophosphoric acid *O,O′*-bis-(2-cyanoethyl) ester *O″*-octadec-9-enyl ester (14 g): Isolated as clear oil (72% yield). ^{1}H NMR ($CDCl_3$): δ 5.31−5.42 (m, 2H), 4.27−4.31 (m, 4H), 4.09−4.21 (m, 2H), 2.78 (t, *J* = 6.0 Hz, 4H), 2.02 (m, 2H), 1.65−1.78 (m, 2H), 1.27 (br s, 22H), 0.88 (t, *J* = 7.2 Hz, 3H); ^{31}P NMR ($CDCl_3$): δ 84.56 (s); MS: [M + ^{23}Na] at *m/z* 493.5.

General Procedure for Preparation of Thiophosphoric Acid *O*-Alkyl/Alkenyl Esters (15a−g). A solution of 100 mg of **14a−g** in 1 M methanolic KOH (10 mL) was stirred for 2 h, and TLC showed the completion of the reaction. The solvent was evaporated to give the crude product, which was dissolved in water (20 mL) and acidified with HCl. The aqueous mixture was extracted with ethyl acetate (2 × 50 mL), and the organic layer was dried over sodium sulfate and concentrated under vacuum to give the desired compound (**15a−g**) as light yellow colored oil.

Thiophosphoric acid *O*-decyl ester (15a): Isolated as light yellow colored oil (80% yield). ^{1}H NMR (DMSO-d_6): δ 3.75−3.95 (m, 2H), 1.50−1.65 (m, 2H), 1.24 (br s, 14H), 0.86 (t, *J* = 6.0 Hz, 3H); ^{31}P NMR (DMSO-d_6): δ 77.50 (s); MS: [M − H]⁻ at *m/z* 253.2. Anal. ($C_{10}H_{23}O_3PS$) C, H.

Thiophosphoric acid *O*-dodecyl ester (15b): Isolated as light yellow colored oil (73% yield). ^{1}H NMR (DMSO-d_6): δ 3.54−3.80 (m, 2H), 1.51−1.64 (m, 2H), 1.24 (br s, 18H), 0.83 (t, *J* = 6.9 Hz, 3H); ^{31}P NMR (DMSO-d_6): δ 78.55 (s); MS: [M − H]⁻ at *m/z* 280.9. Anal. ($C_{12}H_{27}O_3PS$·0.5H_2O) C, H.

Thiophosphoric acid *O*-tetradecyl ester (15c): Isolated as light yellow colored oil (70% yield). ^{1}H NMR (DMSO-d_6): δ 3.78−3.92 (m, 2H), 1.50−1.62 (m, 2H), 1.24(br s, 22H), 0.85 (t, *J* = 6.0 Hz, 3H); ^{31}P NMR (DMSO-d_6): δ 77.54 (s); MS: [M − H]⁻ at *m/z* 309.4. Anal. ($C_{14}H_{31}O_3PS$·0.25H_2O) C, H.

Thiophosphoric acid *O*-dec-9-enyl ester (15d): Isolated as light yellow colored oil (76% yield). ^{1}H NMR (DMSO-d_6): δ 5.70−5.90 (m, 1H), 4.85−5.05 (m, 2H), 3.75−3.98 (m, 2H), 2.01 (q, *J* = 6.6 Hz, 4H), 1.48−1.68 (m, 2H), 1.26 (br s, 10H); ^{31}P NMR (DMSO-d_6): δ 77.75 (s); MS: [M − H]⁻ at *m/z* 251.1. Anal. ($C_{10}H_{21}O_3PS$) C, H.

Thiophosphoric acid *O*-dodec-9-enyl ester (15e): Isolated as light yellow colored oil (80% yield). ^{1}H NMR (DMSO-d_6): δ 5.20−5.38 (m, 2H), 3.85 (q, *J* = 6.6 Hz, 2H), 1.90−2.05 (m, 4H), 1.50−1.62 (m, 2H), 1.26 (br s, 10H), 0.91 (t, *J* = 7.5 Hz, 3H); ^{31}P NMR (DMSO-d_6): δ 79.50 (s); MS: [M − H]⁻ at *m/z* 279.5. Anal. ($C_{12}H_{25}O_3PS$·0.35H_2O) C, H.

Thiophosphoric acid *O*-tetradec-9-enyl ester (15f): Isolated as light yellow colored oil (72% yield). ^{1}H NMR (DMSO-d_6): δ 5.28−5.45 (m, 2H), 3.76−3.96 (m, 2H), 1.90−2.08 (m, 4H), 1.50−1.62 (m, 2H), 1.26 (br s, 14H), 0.86 (t, *J* = 6.9 Hz, 3H); ^{31}P NMR (DMSO-d_6): δ 78.50 (s); MS: [M − H]⁻ at *m/z* 307.5. Anal. ($C_{14}H_{29}O_3PS$·0.3H_2O) C, H.

Thiophosphoric acid *O*-octadec-9-enyl ester (15g): Isolated as light yellow colored oil (82% yield). ^{1}H NMR (DMSO-d_6): δ 5.28−5.42 (m, 2H), 3.75−3.95 (m, 2H), 1.91−2.05 (m, 4H), 1.51−1.62 (m, 2H), 1.24 (br s, 22H), 0.85(t, *J* = 6.9 Hz, 3H); ^{31}P NMR (DMSO-d_6): δ 79.75 (s); MS: [M − H]⁻ at *m/z* 363.5. Anal. ($C_{18}H_{37}O_3PS$·0.3H_2O) C, H.

(1,1-Difluoropentadecyl)-phosphonic Acid Diethyl Ester (18). To a solution of diethyl difluoromethanephosphonate (**17**, 1.0 g, 5.316 mmol) in anhydrous THF (50 mL) was added 2 M LDA (626 mg, 5.847 mmol) at −78 °C, and the mixture was stirred for 30 min. Tetradecyl bromide (1.474 g, 5.316 mmol) in anhydrous THF (10 mL) was added to the mixture at −78 °C, and the reaction mixture was stirred overnight at room temperature. THF was evaporated and the residual oil was purified by flash chromatography using hexanes/ethyl acetate (7:3) as eluent to give 817 mg (40%) of compound **18** as colorless oil. ^{1}H NMR ($CDCl_3$): δ 4.21−4.31 (m, 4H), 1.80−2.10 (m, 2H), 1.51−1.64 (m, 2H), 1.37 (t, *J* = 6.9 Hz, 6H), 1.25 (br s, 22H), 0.87 (t, *J* = 6.6 Hz, 3H); ^{31}P NMR ($CDCl_3$): δ 22.55 (t, *J* = 90.96 Hz); MS: [M + ^{23}Na] at *m/z* 407.2.

(1,1-Difluoro-pentadecyl)-phosphonic Acid (19). To a solution of vacuum-dried **18** (225 mg, 0.585 mmol) in anhydrous methylene chloride (5 mL) was added bromotrimethylsilane (895 mg, 5.85 mmol), and the mixture was stirred at room temperature for 6 h. Solvents were removed under reduced pressure, and the residue was stirred in 95% methanol in water (3 mL) for 1 h. The mixture was concentrated under reduced pressure and dried under vacuum to give 150 mg (78%) of **19** as light yellow solid. mp 66−69 °C; ^{1}H NMR (CD_3OD): δ 1.80−2.06 (m, 2H), 1.42−1.58 (m, 2H), 1.24 (br s, 22H), 0.90 (t, *J* = 6.6 Hz, 3H); ^{31}P NMR (CD_3OD): δ 23.85 (t, *J* = 107.18 Hz); MS: [M − H]⁻ at *m/z* 327.3. Anal. ($C_{15}H_{31}F_2O_3P$·0.2H_2O) C, H.

High Throughput Ca^{2+} Measurements. RH7777 cells stably expressing either LPA_1, LPA_2, or LPA_3[27,33] or PC-3 cells were plated on poly-D-lysine-coated black-wall clear-bottom 96-well plates (Becton Dickinson, San Jose, CA) with a density of 50000 cells/well and cultured overnight. The culture medium (DMEM containing 10% FBS) 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 6−8 h (12 h for PC-3 cells). Cells were loaded with Fura-2 AM for 35 min in modified Krebs medium containing 2% pluronic acid. The dye loading medium was removed and replaced with 100 μL of modified Krebs medium/well prior to analysis in a Flex Station II

instrument. Changes in intracellular Ca^{2+} concentration were monitored by measuring the ratio of emitted light intensity at 520 nm elicited by excitation at 340 or 380 nm wavelength lights, respectively. Each well was monitored for 80−120 s. A 50 μL amount of the test compound (3× stock solution in modified Krebs) was added automatically after 15 s of starting the measurement. Time courses were recorded using the SoftMax Pro software (Molecular Devices, Sunnyvale, CA). Ca^{2+} transients were quantified automatically by calculating the difference between maximum and baseline ratio values for each well.

To evaluate the whether the FAP antagonists are reversible, we applied assay conditions described in our previous publications.[47] Briefly, RH7777 cells stably transfected with LPA GPCR were plated at a density of 4×10^5 on poly-L-lysine-coated glass coverslips in growth medium (DMEM containing 10% FBS) overnight at 37 °C. The normal growth medium was replaced with serum-free DMEM for 6 h before Ca^{2+} measurements. Cells were loaded with Fura-2 AM in serum-free DMEM for 35 min. The coverslips were gently washed twice with Krebs solution in order to remove excess Fura 2-AM. Cells were exposed to LPA, washed for 500 s, and exposed to FAP antagonists at a concentration of 30 μM together with LPA. This concentration completely inhibited LPA-induced Ca-transients. The cells were washed for an additional ∼500 s and reexposed to the original concentration of LPA.

PPARγ Activation Assay. Determination of PPARγ activation in CV1 cells transfected with an acyl-coenzyme A oxidase-luciferase (PPRE-Acox-Rluc) reporter gene construct was run as previously reported.[37] Briefly, CV-1 cells were plated in 96-well plates (5×10^3 cells per well) in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. The next day, the cells were transiently transfected with 125 ng of pGL3-PPRE-Acox-Rluc, 62.5 ng of pcDNAI-PPARγ, and 12.5 ng of pSV-β-galactosidase (Promega, Madison, WI) using LipofectAMINE 2000 (Invitrogen). Twenty-four hours after transfection, cells were treated with 1% FBS supplemented OptiMEMI (Invitrogen) containing DMSO or 10 μM test compound dissolved 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, Foster City, CA), respectively. Samples were run in quadruplicate and the mean ± standard errors were calculated. Data are representative of at least two independent transfections. Student's *t*-test was used for null hypothesis testing, and $P < 0.05$ was considered significant (in the figures $P < 0.05$ is denoted by * and $P < 0.01$ is **).

Isolation of Platelets and Measurement of Platelet Shape Change. For measurement of shape change, human platelets were treated with acetylsalicylic acid and isolated in the presence of apyrase as described previously.[48] Platelets were resuspended at a concentration of $4 \times 10^5/\mu$L in buffer (20 mM HEPES, 138 mM NaCl, 2.9 mM KCl, 1 mM $MgCl_2$, 0.36 mM NaH_2PO_4, 5 mN glucose; 0.6 U ADPase/ml apyrase; pH 7.4). The test compounds were dissolved in methanol (generally at 2 mM concentration) and stored at −80 °C. Before the experiment, methanol was evaporated under a stream of nitrogen, and the lipids were resuspended by sonication in buffer (20 mM HEPES, 138 mM NaCl, BSA 0.25 mM) to obtain a 1 mM solution (with a ratio of lipid/albumin of 4:1). Suspensions of washed platelets were transferred into aggregometer cuvettes, incubated at 37 °C with stirring (1100 rpm), and exposed to various concentrations of the compounds or vehicle control. After a fixed incubation time, LPA 16:0 was added. Shape change was measured by the decrease in light transmission of the stirred platelet suspension in a LABOR aggregometer (Fresenius, Bad Homburg, Germany).

Autotaxin Assay. Autotaxin phosphodiesterase activity was determined using the fluorescence-resonance energy-based sensor CPF4[49] as a substrate, as described by van Meeteren et al.[38] Briefly, recombinant ATX in Tris-buffered saline (140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 50 mM Tris; pH 8.0) was incubated with or without the test compounds, and CPF4 was added at a concentration of 2 μM. CPF4 fluorescence was monitored (at 37 °C) in a BMG Fluorstar 96-well plate reader (excitation at 355 nm; emission at 460 and 520 nm).

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Supporting Information Available: Table S1, listing the elemental analyses of the final compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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