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## Dual Activity Lysophosphatidic Acid Receptor Pan-Antagonist/Autotaxin Inhibitor Reduces Breast Cancer Cell Migration *In vitro* and Causes Tumor Regression *In vivo*

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### Abstract

Signal transduction modifiers that modulate the lysophosphatidic acid (LPA) pathway have potential as anticancer agents. Herein, we describe metabolically stabilized LPA analogues that reduce cell migration and invasion and cause regression of orthotopic breast tumors *in vivo*. Two diastereoisomeric  $\alpha$ -bromophosphonates (BrP-LPA) were synthesized, and the pharmacology was determined for five LPA G protein-coupled receptors (GPCRs). The *syn* and *anti* diastereomers of BrP-LPA are pan-LPA GPCR antagonists and are also nanomolar inhibitors of the lysophospholipase D activity of autotaxin, the dominant biosynthetic source of LPA. Computational models correctly predicted the diastereoselectivity of antagonism for three GPCR isoforms. The *anti* isomer of BrP-LPA was more effective than *syn* isomer in reducing migration of MDA-MB-231 cells, and the *anti* isomer was superior in reducing invasion of these cells. Finally, orthotopic breast cancer xenografts were established in nude mice by injection of MB-231 cells in an *in situ* cross-linkable extracellular matrix. After 2 weeks, mice were treated with the BrP-LPA alone (10 mg/kg), Taxol alone (10 mg/kg), or Taxol followed by BrP-LPA. All treatments significantly reduced tumor burden, and BrP-LPA was superior to Taxol in reducing blood vessel density in tumors. Moreover, both the *anti*- and *syn*-BrP-LPA significantly reduced tumors at 3 mg/kg.

### Introduction

Lipid signaling in cancer is dominated by the signal transduction through two pathways involving overproduction of two phosphorylated lipids: phosphatidylinositol 3,4,5-trisphosphate (PIP<sub>3</sub>) and lysophosphatidic acid (LPA). In both pathways, down-regulated tumor suppressors and up-regulated oncogenes result in a systemic or local overproduction of these potent agonists, and these defects are present in many advanced cancers. Quite recently, therapeutically important inhibitors of the phosphatidylinositol 3-kinase (1)

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**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

#### Disclosure of Potential Conflicts of Interest

G. Tigyi is an equity holder in RxBio. G.D. Prestwich is an equity holder in Glycosan BioSystems and advisor to RxBio and Echelon Biosciences. The other authors declared no potential conflicts of interest.

responsible for PIP<sub>3</sub> production have entered the clinic (2). However, anticancer agents that modify signaling through the LPA signaling pathway (3–6) remain in preclinical research, although the molecular understanding of pathway has greatly increased (7).

LPA signaling occurs via six receptors belonging to the seven transmembrane G protein-coupled receptor (GPCR) protein family, now LPA<sub>1</sub> to LPA<sub>6</sub>. The first three, LPA<sub>1</sub> to LPA<sub>3</sub>, formerly EDG2, EDG4, and EDG7 (8), are expressed from endothelial differentiation genes (*edg*). The recently orphaned receptors GPR23/P2Y9 (9), GPR92 (10), and GPR87 (11), designated as LPA<sub>4</sub>, LPA<sub>5</sub>, and LPA<sub>6</sub>, belong to the purinergic cluster of GPCRs. Cancer cells of different cellular origins express LPA GPCRs in differing amounts, e.g., LPA<sub>1</sub> is widely expressed in cancer cells, whereas LPA<sub>4</sub> is expressed at low levels. Ovarian and breast cancer cells express multiple LPA GPCR isoforms, and LPA accumulates in tumor cell as cisternae and effusates (4, 5).

LPA elicits growth factor-like effects in most cell types. LPA is implicated in physiologic responses that include immunologic competence, brain development, wound healing, coagulation, and regulation of blood pressure. The pleiotropic physiologic functions of LPA contribute to pathophysiologic states that include cancer, atherosclerosis, hypertension, ischemia reperfusion injury, and prevention of radiation-induced toxicity (12). As GPCRs, LPA receptors should be amenable to the development of highly specific and potent agonists or antagonists. Validated compounds are essential to advance *in vivo* studies and to minimize off-target effects. To address this need, we developed bioactive analogues of LPA that resist phosphatase, acyl transferase, and lipase activities (13, 14).

LPA and its structural analogues all have a polar head group, a linker, and a hydrophobic tail (Fig. 1A). Hydrolysis of the phosphate head group of LPA by ubiquitous phosphatases deactivates LPA, but modifications at this site are the least well tolerated. Stable phosphomimetics that are micromolar or submicromolar LPA agonists include phosphonates, e.g.,  $\alpha$ -hydroxymethylene phosphonates (15) and  $\alpha$ -fluoromethylene phosphonates (16).

Targeting the biosynthesis of LPA is also a valid target for therapeutic development. The lysophospholipase D (lysoPLD) activity of the tumor-associated protein autotaxin (ATX) converts lysophosphatidylcholine (LPC) to LPA (17, 18). ATX is the product of 1 of the 40 most up-regulated genes in invasive cancers and is implicated in cell motility and tumor invasion, metastasis, and neovascularization (19, 20). ATX is feedback inhibited by its product LPA (21). Thus, the ideal anticancer drug targeting LPA signaling would simultaneously abrogate signaling through the GPCRs and attenuate LPA production by ATX (5). Indeed, we previously observed that the palmitoyl  $\alpha$ -bromomethylenephosphonate **1** (Fig. 1A; BrP-LPA) was a pan-antagonist for LPA<sub>1</sub>, LPA<sub>2</sub>, LPA<sub>3</sub>, and LPA<sub>4</sub> GPCRs (15). This diastereomeric mixture also inhibited over 98% of ATX activity at 10  $\mu$ mol/L.

Herein, we describe the enantioselective syntheses of the *syn* and *anti* diastereomers of BrP-LPA. Computational modeling of the *syn* and *anti* diastereomers predict the independently determined *in vitro* pharmacology and cell biology of these diastereomers. Next, we show that each diastereoisomer inhibits ATX and inhibit cell migration and invasion. Finally, treatment with BrP-LPA diastereomers causes tumor regression and decreased tumor vascularity *in vivo* in an orthotopic breast cancer xenograft model (22, 23).

## Materials and Methods

### Chemical synthesis

Full experimental details can be found in the Supplementary Data. The final products are described below and summarized in Fig. 1B.

- a. 1(*S*)-Bromo-3(*S*)-hydroxy-4-(palmitoyloxy)butyl]phosphonate (**1b**). To a solution of phosphonate **10b** (120 mg, 0.18 mmol) in 8 mL EtOAc was added PtO<sub>2</sub> (6 mg). The mixture was stirred under 1 atm H<sub>2</sub> atmosphere for 20 min, filtered, and concentrated to give *anti* isomer **1b** (87 mg, 100%). [ $\alpha$ ]<sub>20</sub><sup>D</sup> = -32.1 (*c* 0.5, CHCl<sub>3</sub>); <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  4.11-4.00 (m, 4H), 2.28 (t, *J* = 7.6 Hz, 2H), 2.15-2.08 (m, 1H), 1.94-1.85 (m, 1H), 1.63-1.56 (m, 2H), 1.18 (m, 24H), 0.80 (t, *J* = 7.2 Hz, 3H); <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>)  $\delta$  174.6, 68.1, 66.8, 66.7, 40.9, 39.3, 36.6, 34.2, 32.1, 29.8, 29.78, 29.7, 29.6, 29.5, 29.4, 29.3, 25.0, 22.8, 14.1; <sup>31</sup>P NMR (162 MHz, CDCl<sub>3</sub>)  $\delta$  20.21 (1P); MALDI-HRMS [M + Na]<sup>+</sup> calcd for C<sub>20</sub>H<sub>40</sub>BrO<sub>6</sub>PNa 509.1638, 511.1621, found 509.1634, 511.1557.
- b. 1(*R*)-Bromo-3(*S*)-hydroxy-4-(palmitoyloxy)butyl]phosphonate (**1a**) was obtained from **10a** in 94% yield analogously, as described for compound **1b**. [ $\alpha$ ]<sub>20</sub><sup>D</sup> = +3.2 (*c* 0.5, CHCl<sub>3</sub>); <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  4.13 (m, 1H), 4.05-4.00 (m, 2H), 3.95-3.98 (m, 1H), 2.28 (t, *J* = 7.6 Hz, 2H), 2.25-2.08 (m, 2H), 1.54 (m, 2H), 1.18 (m, 24H), 0.80 (t, *J* = 6.8 Hz, 3H); <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>)  $\delta$  174.6, 67.6, 67.5, 67.1, 39.5, 37.9, 37.4, 34.2, 32.1, 29.8, 29.78, 29.7, 29.6, 29.5, 29.4, 29.3, 24.98, 22.8, 14.1; <sup>31</sup>P NMR (162 MHz, CDCl<sub>3</sub>)  $\delta$  19.64 (1P); MALDI-HRMS [M + Na]<sup>+</sup> calcd for C<sub>20</sub>H<sub>40</sub>BrO<sub>6</sub>PNa 509.1638, 511.1621, found 509.1639, 511.1605.

### Receptor activation

Assays for mobilization of intracellular Ca<sup>2+</sup> were performed as described (15, 24), with details in the Supplementary Data. Each test was performed in quadruplicate. EC<sub>50</sub>, IC<sub>50</sub>, and *K*<sub>i</sub> values were calculated by fitting a sigmoid function to data points (25).

### Molecular modeling

Docking simulations were performed using Autodock 3.0 (26). The compounds were constructed using MOE with a -2 charge on the phosphate, and geometry was optimized with MMFF94 (27). Each compound was flexibly docked in inactive models (28) of LPA<sub>1-3</sub>. A mol2 format file of the receptor consisting of the structure coordinates with MMFF94 charges was generated in MOE. The receptor input file was prepared using the molto2pdbqs utility, and ligands were prepared with Autotors. A docking box was defined to encompass the binding site (28). Default parameters of Autodock 3.0 were used, except for energy evaluations ( $9 \times 10^{10}$ ), genetic algorithm search generations ( $3 \times 10^4$ ), maximum local search iterations ( $3 \times 10^3$ ), and 15 runs. A single complex was selected for each ligand-receptor combination based on both rank and proximity of ligand phosphate group to R3.28. Usually, the top-ranked structure showed close interaction with R3.28 and was selected. For **1a**-LPA<sub>3</sub>, the second-ranked structure was selected. The selected complexes were minimized with MMFF94 and interactions were analyzed.

### ATX assay

Using FS-3 (ref. 29; Echelon Biosciences, Inc.) as substrate and recombinant hemagglutinin-tagged ATX (ATX-HA), 50  $\mu$ L of ATX-HA (0.25  $\mu$ g) in assay buffer [Tris 50 mmol/L, NaCl 140 mmol/L, KCl 5 mmol/L, CaCl<sub>2</sub> 1 mmol/L, MgCl<sub>2</sub> 1 mmol/L (pH 8.0)] was mixed with 25  $\mu$ L of FS-3 (final, 1  $\mu$ mol/L) and 25  $\mu$ L of test compound dissolved in assay buffer

with 1:1.5 bovine serum albumin in 96-well plate. FS-3 fluorescence at excitation and emission wavelengths of 485 and 538 nm, respectively, were monitored using a FLEX station II for 2 h of incubation at 37°C. The differences between time of 0 and 120 min were calculated individually and normalized to the vehicle control. The mean  $\pm$  SD of triplicate sample wells was expressed as percentage of ATX inhibition. Significant difference compared with vehicle was determined by the Student's *t* test at a *P* value of 0.05 (25).

### Scratch wound assay

MDA-MB-231 cells were plated in triplicate into six-well plates at  $3 \times 10^5$  per well. At 48 h, the confluent cell layer was scratched using a sterile pipette tip. Nonadherent cells and cellular debris were removed by washing (PBS). Fresh medium containing BrP-LPA **1**, *syn*-BrP-LPA **1a**, or *anti*-BrP-LPA **1b** (1–100  $\mu$ mol/L) was added to the scratched monolayers. Cells were observed and digitally photographed. Inhibition of migration was assessed and quantified by using ImageJ (30).

### Xenograft establishment and chemotherapy

Female *nu/nu* mice (ages, 4–6 wk; Charles River Laboratories) were anesthetized by i.p. injection of ketamine (80 mg/kg) and xylazine (10 mg/kg), as approved by the University of Utah Institutional Animal Care and Use Committee. Before inoculation, MDA-MB-231 cells were trypsinized and resuspended in Extracel (Glycosan BioSystems) with a final concentration of  $5 \times 10^7$  cells/mL, and the resulting suspension was mixed gently. An aliquot of 200  $\mu$ L of the mixture was injected s.c. into the fourth mammary fat pad of each mouse. The mice were randomly divided into treatment groups and control groups (six mice per group). Treatments were Taxol (10 mg/kg), BrP-LPA **1** (10 mg/kg), or Taxol (10 mg/kg) followed by BrP-LPA **1** (10 mg/kg). The control was physiologic saline. Injections (i.p.) were performed twice per week for 2 wk, starting 2 wk after the cell transplantation. Tumor sizes were measured and calculated: tumor size ( $\text{mm}^3$ ) = [width (mm)]<sup>2</sup>  $\times$  [length (mm)]/2. After sacrifice, tumor tissue was removed for histological H&E and immunohistochemistry using an anti-CD31 antibody. CD31 in zinc-fixed paraffin sections was detected using antirat immunoglobulin horseradish peroxidase detection kit (BD Bioscience). Microvessels were counted at  $\times 400$  magnification, and the data were converted to microvessel density (vessels/ $\text{mm}^2$ ), with 1 microscopic field = 0.196  $\text{mm}^2$ . Six fields were randomly chosen for quantification in three slides for each treatment group tumor tissue.

The effect of *syn*-BrP-LPA **1a** and *anti*-BrP-LPA **1b** were evaluated separately after an analogous protocol. The cell suspension injection volume was reduced to 100  $\mu$ L, and dosages were 3 mg/kg for **1a** and **1b**.

### Statistical methods

Data from *in vitro* and *in vivo* experiments are expressed as the mean  $\pm$  SD of at least triplicate determinations. Statistical comparisons were performed by Student's *t* test, and differences were considered significant at *P* < 0.05.

## Results

### Diastereoselective synthesis and absolute stereochemistry

The synthesis of the individual diastereoisomers **1a** (*syn*) and **1b** (*anti*) used a stereocontrolled bromination of the separated  $\alpha$ -hydroxymethylene phosphonates (Fig. 1B). Aldehyde **4** was prepared in two steps in high yield from acetone **3** and converted to the diastereomeric mixture of  $\alpha$ -hydroxymethylene phosphonates **5a** and **5b**. Although this mixture was not readily separable, the TES ethers **6a** and **6b** could be separated by flash

chromatography, and the silyl group could be readily removed. Using purified isomer **5b**, bromination was performed at high dilution (1 g of **5b** in 80 mL) in 12:1 toluene:pyridine to optimize the yield (Fig. 1B). After acetonide deprotection, selective palmitoylation, and hydrogenation, the pure *anti* diastereomer **1b** was obtained in quantitative yield. The *syn* diastereoisomer **1a** was obtained similarly, starting with **5a**.

The stereochemical assignment of the absolute configuration of  $\alpha$ -position was accomplished by two-dimensional NMR analysis of 1,3-diol acetonide (31, 32). Thus, isomer **6b** was converted in several steps (deprotection, selective silylation, acetonide formation) to acetonide **7b** (Supplementary Fig. S1). The absolute configuration of the  $\alpha$ -C in **7b** was confirmed by coupling constants and  $^1\text{H}$ - $^1\text{H}$  NOESY. Specifically, NOEs were observed between  $\text{H}_1$  and  $\text{H}_3$ ,  $\text{H}_1$  and  $\text{H}_{2e}$ , and  $\text{H}_3$  and  $\text{H}_{2e}$ , establishing a *syn*-1,3-relationship in phosphonate **7b**. In **7a**, no NOEs were observed between  $\text{H}_1$  and  $\text{H}_3$ , confirming the *anti*-1,3 relative stereochemistry. In addition, the acetonide methyls of **7b** displayed  $^{13}\text{C}$  NMR chemical shifts characteristic of the acetonide of a *syn*-1,3-diol (31).

### Receptor activation assays

The ligand properties of the compounds were evaluated using a  $\text{Ca}^{2+}$  mobilization assay to assess activation or inhibition of  $\text{LPA}_1$ ,  $\text{LPA}_2$ ,  $\text{LPA}_3$ , and  $\text{LPA}_5$  expressed in RH7777 cells and  $\text{LPA}_4$  expressed in Chinese hamster ovary (CHO) cells. Table 1 illustrates calcium responses elicited through the response of human  $\text{LPA}_1$ ,  $\text{LPA}_2$ ,  $\text{LPA}_3$ ,  $\text{LPA}_4$ , and  $\text{LPA}_5$  receptors to *syn*-BrP-LPA **1a**, *anti*-BrP-LPA **1b**, and BrP-LPA **1**. These cell lines are used extensively for characterization of LPA GPCR ligands, because RH7777 cells are intrinsically unresponsive to LPA, whereas wild-type CHO cells show minimal endogenous responses to LPA (5, 9, 25, 33).

The diastereomeric mixture BrP-LPA **1** (prepared from unseparated isomers **5a** and **5b**) showed pan-antagonist activity for stably transfected receptors  $\text{LPA}_{1-4}$  submicromolar potency toward  $\text{LPA}_2$ , modest inhibition of LPA activation of  $\text{LPA}_4$ , and weak partial agonism for the transiently transfected  $\text{LPA}_5$ . The *syn*-BrLPA **1a** was a pan-antagonist for the *edg* family GPCRs, showing  $K_i$  values of 273, 250, and 1830 nmol/L for  $\text{LPA}_1$ ,  $\text{LPA}_2$ , and  $\text{LPA}_3$ , respectively. For the purinergic family GPCRs, *syn*-BrLPA **1a** was an antagonist for  $\text{LPA}_4$  but a weak agonist for  $\text{LPA}_5$ . Apparently, the overall agonist effect dominates in the mixed diastereomers. By comparison, *anti*-BrP-LPA **1b** was a pan-antagonist for the *edg* family GPCRs, with  $K_i$  values of 752, 241, and 623 nmol/L for  $\text{LPA}_1$ ,  $\text{LPA}_2$ , and  $\text{LPA}_3$ , respectively. In addition, *anti*-BrP-LPA **1b** was an antagonist for the two purinergic family GPCRs, with partial antagonism of LPA on  $\text{LPA}_4$  and a  $K_i$  value of 376 nmol/L for  $\text{LPA}_5$ .

### Molecular modeling of diastereoisomers with $\text{LPA}_1$ , $\text{LPA}_2$ , and $\text{LPA}_3$

Molecular models of the complexes formed by *syn*-BrP-LPA **1a** and *anti*-BrP-LPA **1b** with  $\text{LPA}_{1-3}$  were created without prior knowledge of the pharmacologic results. The models showed ionic interactions between the **1a** or **1b** phosphate groups and basic residues in the  $\text{NH}_2$  terminus, third transmembrane domain, and seventh transmembrane domain in  $\text{LPA}_{1-3}$  (Fig. 2). The basic  $\text{NH}_2$  terminal residues showed variable position and type, with  $\text{LPA}_1$  interacting via R36,  $\text{LPA}_2$  interacting via H91, and  $\text{LPA}_3$  interacting via K95. The interacting residue in the third transmembrane domain is the conserved arginine appearing at position 3.28, a residue that is critical for both LPA recognition by EDG family LPA receptors (34–36) and S1P recognition by the EDG family S1P receptors (36, 37). The interacting residue in the seventh transmembrane domain of  $\text{LPA}_1$  and  $\text{LPA}_2$  is K7.36, whereas  $\text{LPA}_3$  uses R7.36. Moreover, K7.35 in  $\text{LPA}_3$ , not R7.36, is essential for LPA recognition, although this lysine is replaced by an acidic residue in  $\text{LPA}_1$  and  $\text{LPA}_2$  (36). The hydrophobic tails interact primarily with residues in the extracellular loops, consistent

with previous models of LPA receptor antagonism (24, 25, 28). The geometries of these complexes accurately predicted that both *syn*-BrP-LPA **1a** and *anti*-BrP-LPA **1b** would antagonize LPA action at all three EDG family LPA receptors by competing for interaction at R3.28 and without independent agonist activity due to their lack of hydrophobic interactions within the transmembrane domain.

### Inhibition of ATX

The inhibition of ATX by BrP-LPA **1**, *syn*-BrP-LPA **1a**, and *anti*-BrP-LPA **1b** was measured at concentrations of 0.01 to 10  $\mu\text{mol/L}$  and compared with the ATX inhibitory effects of 10  $\mu\text{mol/L}$  LPA (18:1) and 10  $\mu\text{mol/L}$  2ccLPA (16:1; ref. 38; Supplementary Fig. S2). ATX activity was measured by the hydrolysis of the fluorogenic lysoPC analogue **FS-3**, with a  $K_m$  value of 6.3  $\mu\text{mol/L}$  (29). Preliminary results showed that each analogue inhibited >98% of ATX at 10  $\mu\text{mol/L}$ . A clear dose-response effect on inhibition was observed for both *syn*-BrP-LPA **1a** ( $\text{IC}_{50} = 165 \text{ nmol/L}$ ) and *anti*-BrP-LPA **1b** ( $\text{IC}_{50} = 22 \text{ nmol/L}$ ; Supplementary Fig. S2).

### Inhibition of migration

BrP-LPA can both significantly reduce LPA production and block activation of cell surface LPA receptors. Such a molecule has clear therapeutic potential to reduce LPA-induced cell migration, invasion, and proliferation (5, 38, 39). Thus, we evaluated the effect of the analogues on MDA-MB-231 breast cancer cells using a scratch wound assay (Fig. 3A). The relative expression of LPA GPCRs in MDA-MB-231 cells is  $\text{LPA}_1 > \text{LPA}_2 \gg \text{LPA}_3$ , and  $\text{LPA}_1$  seems to be the key in mediating cell migration of normal and neoplastic cells (40). After treatment with pure diastereomers **1a** and **1b** and the mixture BrP-LPA **1**, cells were allowed to migrate into the denuded area for 0, 16, and 24 hours. By 24 hours, untreated control cells completely filled the scratched area. Treatment with *syn*-BrP-LPA **1a** and *anti*-BrP-LPA **1b** at 10 and 40  $\mu\text{mol/L}$  inhibited the MDA-MB-231 cell migration (Fig. 3B). Migration of MB-231 cells was decreased by 57% ( $P < 0.05$ ) by 40  $\mu\text{mol/L}$  *anti*-BrP-LPA **1b** when compared with the untreated control. The mixed diastereomers showed intermediate inhibition of cell migration (data not shown).

### Inhibition of invasion

Using an *in vitro* invasion assay, MDA-MB-231 cells invaded through Matrigel-coated transwell membranes (Supplementary Fig. S3). Treatment with *anti*-BrP-LPA **1a** or *syn*-BrP-LPA **1b** inhibited the invasion by ~52% ( $P < 0.05$ ), but the potencies of the two isomers were not significantly different. This suggests that connecting the complex biology of invasion to specific aspects of pharmacology of individual receptors or ATX inhibition will require additional investigation.

### Tumor regression in engineered orthotopic breast tumor xenografts

To evaluate BrP-LPA *in vivo*, we used “tumor engineering” to create orthotopic breast tumors in nude mice (22, 23). In the first experiment, we compared BrP-LPA **1** alone with Taxol alone. We also simulated a dual-drug therapy approach by administering Taxol followed by the mixed diastereomers **1**. Thus, s.c. mammary fat pad injection of MDA-MB-231 cells suspended in a semisynthetic extracellular matrix (sECM, Extracel) in *nu/nu* mice resulted in tumor growth at each injection site. Figure 4 shows the increase in tumor volumes during the growth phase and decrease in tumor volumes during the treatment phase. During the first 2 wk, the tumor cells expand within the volume of the injected sECM. The gross size of the bolus changes little, but the tumor cell density increases dramatically as cells proliferate, degrade the sECM, and secrete their own ECM. After 2 weeks of tumor growth, the control group was treated with four i.p. injections of physiologic saline over the

course of 2 weeks. The first treatment group received i.p. injections of Taxol (10 mg/kg), and the second treatment group received i.p. injections of BrP-LPA **1** (10 mg/kg), twice per week for 2 weeks. The third treatment group received two injections of Taxol (10 mg/kg) for week 1 and two injections of BrP-LPA **1** (10 mg/kg) for week 2. In each of the three treatment groups, a reduction of tumor size relative to controls was observed shortly after the first therapeutic injection (Fig. 4A).

To examine the separate effects of the two diastereomers, *syn*-BrP-LPA **1a** and *anti*-BrP-LPA **1b**, a second xenograft study was performed using analogous protocols, except that the size of the injected cell suspension was reduced to 100  $\mu$ L and the treatment dosage was reduced to 3 mg/kg. Figure 4B shows that, although each isomer significantly decreased the tumor volume relative to the control group ( $P < 0.01$ ), there was no significant difference between the tumor reduction by **1b** relative to **1a** for this experimental group size ( $P = 0.16$ ). As observed in the invasion assay, the *in vivo* biology can often be more complex than the sum of individual receptor pharmacology and enzyme inhibition outcomes.

After completion of the 2-week treatment course for the study in Fig. 4A comparing the Taxol and BrP-LPA treatments, tumors in each treatment group were significantly decreased or undetectable. At necropsy, tumors were surgically removed and prepared for histologic analysis. The largest tumor in the treatment group with diastereomeric mixture **1** was significantly smaller than the smallest tumor in the control group (Fig. 5A). H&E staining revealed an irregular arrangement of tumor cells, inflammatory granuloma tissue, and an increased number of blood vessels (Fig. 5B) in the control group. An endothelial layer covering tumor vasculature was observed using immunohistochemical staining with anti-CD31 antibody (Fig. 5C). Quantification of the newly generated vessels in the tumor samples in six different fields of three slides for each treatment group (Fig. 5D) showed highly significant reduction of angiogenesis in the mice treated with the LPA antagonist mixture **1** relative to the controls or either of the Taxol treatments ( $P < 0.01$ ).

## Discussion

### Computational modeling predicts pharmacology

The docking of *syn*-BrP-LPA **1a** and BrP-LPA **1b** with GPCRs LPA<sub>1-3</sub> were performed computationally without knowledge of the pharmacologic outcomes. The predictions from the models were gratifyingly congruent with the relative antagonist activity at LPA<sub>1-3</sub>, lending credibility to the predictive power of the model. Distances between phosphate groups of **1a** and **1b** and basic residues in each LPA receptor model provide a molecular context for the relative potencies observed experimentally (Fig. 2; Supplementary Table 1). For example, the phosphate of **1a** interacts more closely with the cationic residue in transmembrane domain 3, consistent with greater antagonism. The interaction distances for **1a** and **1b** with LPA<sub>2</sub> are essentially identical, consistent with equivalent potencies. Whereas both compounds show similar phosphate basic residue distances in LPA<sub>3</sub>, the partially negative bromine atom of **1b** is only 2.5 Å from K95. In contrast, the bromine atoms in the other five complexes are >3.5 Å from the basic residues, consistent with the more potent antagonism of **1b** compared with **1a**.

### Unique LPA antagonists

To our knowledge, this constitutes the first comprehensive pharmacologic assessment of novel analogues of LPA on five LPA GPCRs from two families. Notably, diastereomer **1b** is the first known pan-antagonist, blocking LPA activation at LPA<sub>1-5</sub>. This represents a proof of concept that compounds capable of inhibiting both the *edg* family of LPA receptors (LPA<sub>1-3</sub>) and the purinergic family of LPA receptors represented by LPA<sub>4</sub> and LPA<sub>5</sub> exist.

### BrP-LPA diastereomers differentially inhibit ATX and cell migration

LPA is produced from LPC by ATX/lysoPLD (17, 18, 41), and local production of LPA can support invasion of tumor cells, promoting metastasis (4, 19, 42) via several molecular mechanisms. First, activation of the Rho and Rac GTPase pathways downstream from LPA-GPCRs regulates actin cytoskeleton and cell motility (43). Second, LPA modulates the activity of matrix metalloproteinases, which are crucial for metastasis and LPA-induced transphosphorylation of the epidermal growth factor receptor (44). Third, the potent mitogenic effects of ATX are lead to enhanced angiogenesis (45). Thus, LPA receptor antagonists and ATX inhibitors have potential in cancer therapy both by blocking the growth-supporting, angiogenic, and antiapoptotic effects of LPA and by reducing its titer (42, 46).

Addition of BrP-LPA diastereomers **1a** and **1b** at 40  $\mu\text{mol/L}$  both significantly decreased migration of MDA-MB-231 cells into a scratched monolayer. The more potent ATX inhibitor *anti*-BrP-LPA **1b** showed greater potency in reducing cell migration, although the isomers were equipotent in decreasing the cell invasion through Matrigel-coated transwell membranes.

### BrP-LPA diastereomers cause tumor regression and reduced angiogenesis

Finally, the BrP-LPA diastereomers were evaluated in a xenograft model using engineered orthotopic breast tumors in nude mice (22, 23). This new model has potential for creating vascularized, orthotopic tumors from patient-derived samples and offers a method to introduce labeled metastatic cells into any organ (47). We first used the mixed diastereomers **1** to compare buffer injection with 10 mg/kg i.p. doses, BrP-LPA alone (four doses), Taxol alone (four doses), and Taxol (two doses) followed by BrP-LPA (two doses). The tumors in all treatment groups were significantly decreased. Interestingly, quantification of the newly generated blood vessels in the tumor samples showed highly significant reduction of angiogenesis in the mice treated with BrP-LPA **1** relative to either the controls or Taxol treatments ( $P < 0.01$ ). To rationalize this observation that the Taxol followed by BrP-LPA has the same effect as Taxol alone, we note that Taxol is a mitotic poison, whereas BrP-LPA inhibits signaling pathways important for proliferation, migration, and angiogenesis; this compound likely requires actively dividing cells to exert its effect. Apparently, in tumors with cells in a growth arrested/apoptotic state, no additional effect of the BrP-LPA was possible.

This result is extremely encouraging in view of the requirement for ATX in blood vessel formation during embryogenesis (48) and the growing potential of antiangiogenic drugs, e.g., Avastin, in cancer therapy. In addition, each diastereomer significantly decreased the tumor volume relative to the control group ( $P < 0.01$ ) at 3 mg/kg. However, with six mice per group, only a trend was evident to suggest that *anti*-BrP-LPA **1b** might be more higher efficacy relative to *syn*-BrP-LPA **1a** ( $P < 0.1$ ). The mechanistic basis for the antiangiogenic effects of the BrP-LPA isomers are the subject of active studies with other tumor cells and other matrices.

### Therapeutic potential of ATX inhibitors and LPA antagonists in cancer treatment

LPA GPCRs and ATX present two promising, druggable, yet under exploited targets for cancer therapy (5, 6). ATX, a potent mitogen in metastatic cancers (19) produces a continuous output of LPA (17, 18), potentially abrogating the action of an LPA antagonist. By blocking ATX, the autocrine/paracrine loop that involves the ATX-mediated production of LPA is reduced (42, 46). Although ATX is product feedback inhibited (21), using LPA to inhibit ATX would be counterproductive. Thus, analogues of LPA that retained ATX inhibitory activity, yet did not activate LPA GPCRs, are needed. Recently, a structure-



function study of carbacyclic analogues of cyclophosphatidic acid (ccPA) showed that these compounds were selective inhibitors of ATX and lacked agonist activity for LPA<sub>1,2,3</sub> (38). Importantly, ccPA inhibited cancer cell invasion *in vitro* and suppressed metastasis of melanoma cells *in vivo*. However, ATX inhibition alone was inadequate to accomplish both dampen LPA production and suppress receptor activation by endogenous LPA. In a separate study, different small-molecule ATX inhibitors were found to reduce melanoma cell migration *in vitro* (39).

In conclusion, we established that stereoisomers of BrP-LPA, in particular *anti*-BrP-LPA **1b**, simultaneously inhibited ATX and antagonized five of the known LPA GPCRs *in vitro*. Moreover, we established *in vivo* efficacy of the targeted polypharmacology (49) concept for the LPA pathway by showing the regression and reduction of angiogenesis in tumor xenografts. Taken together, these data provide the first proof of concept for the use of a dual-function pan-LPA receptor antagonist and ATX inhibitor in cancer chemotherapy.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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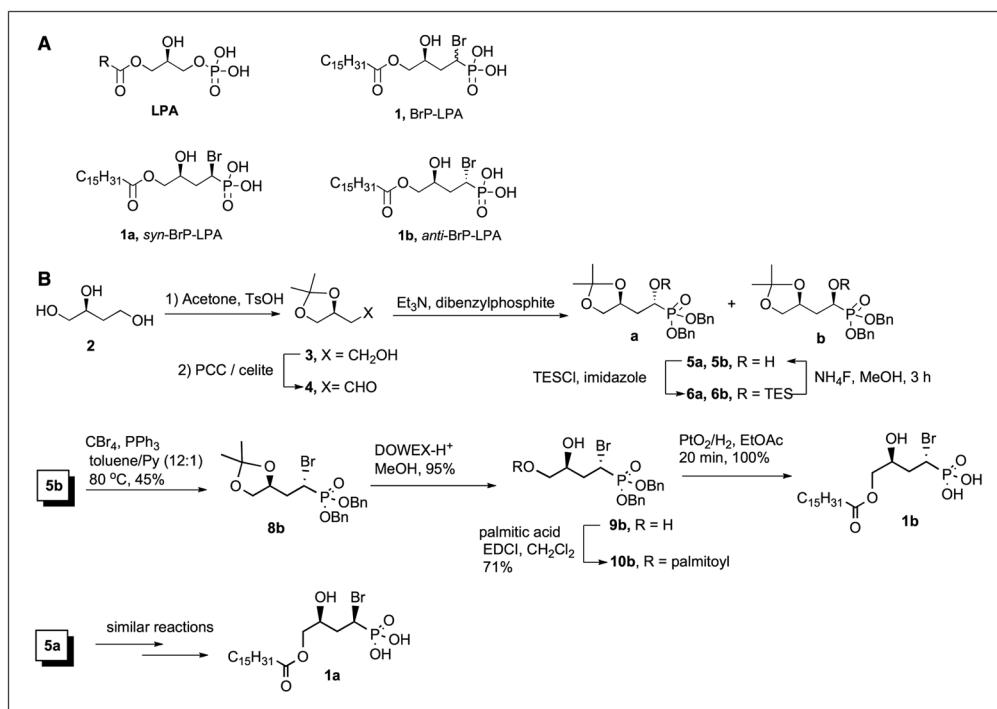
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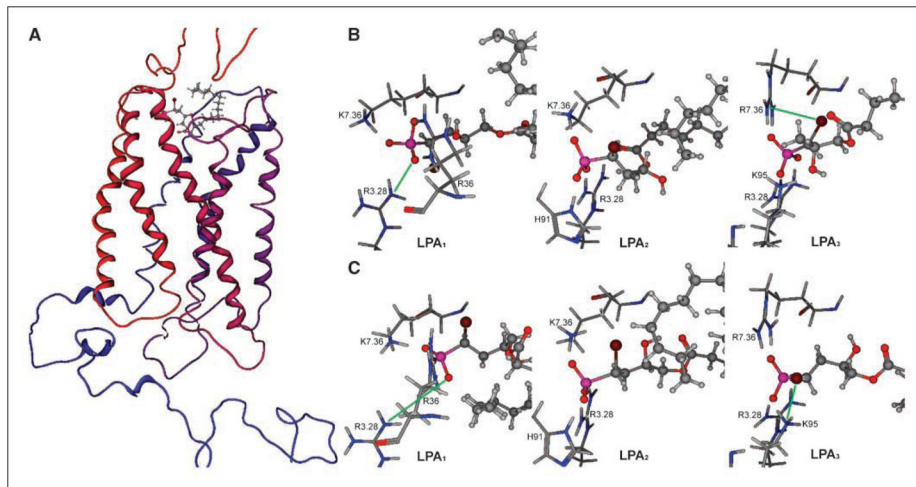
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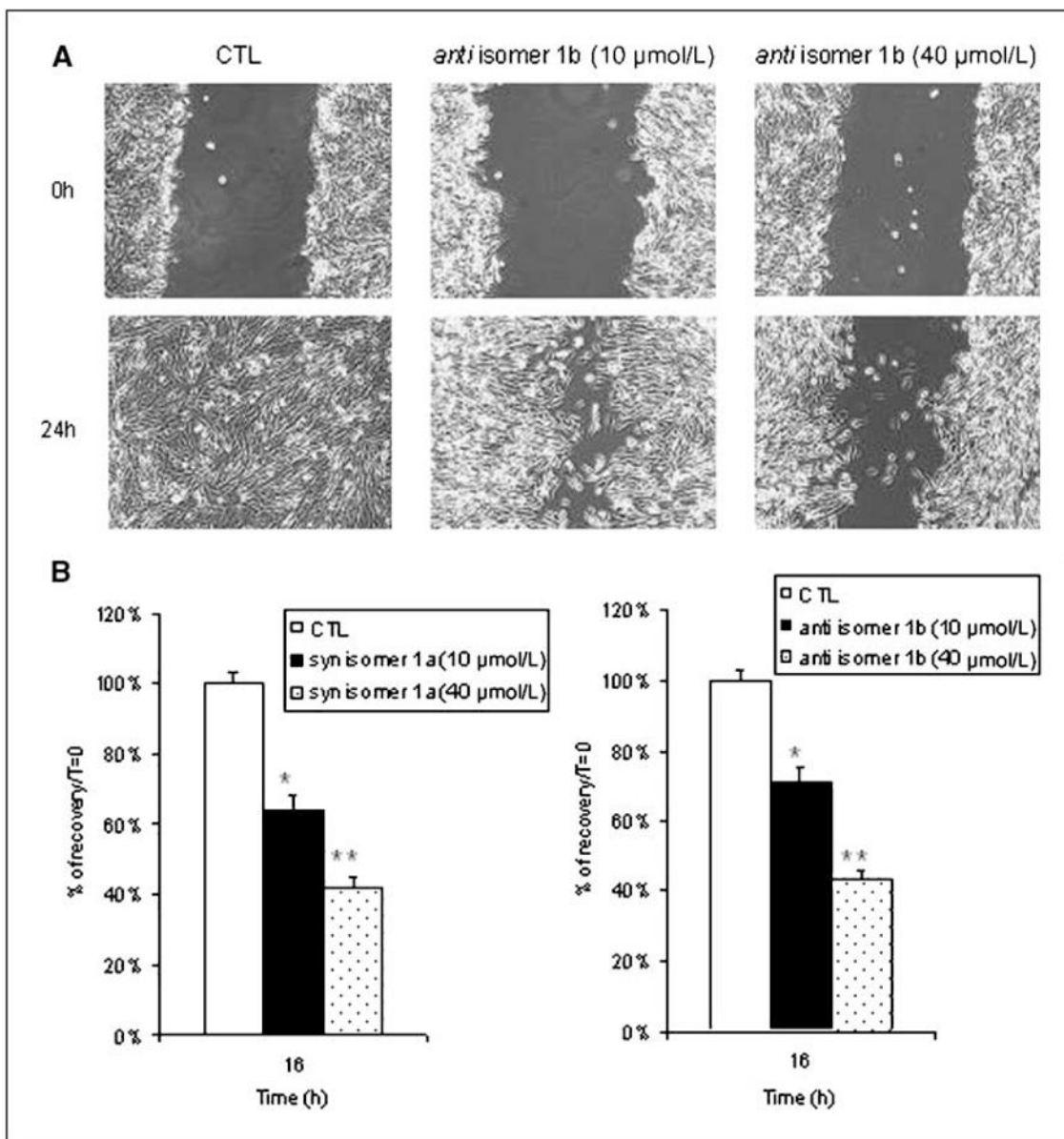
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**Figure 1.** *A*, structures of LPA and stabilized analogues. *B*, synthesis of *syn*- and *anti*-BrP-LPA isomers **1a** and **1b**. See text for experimental details.

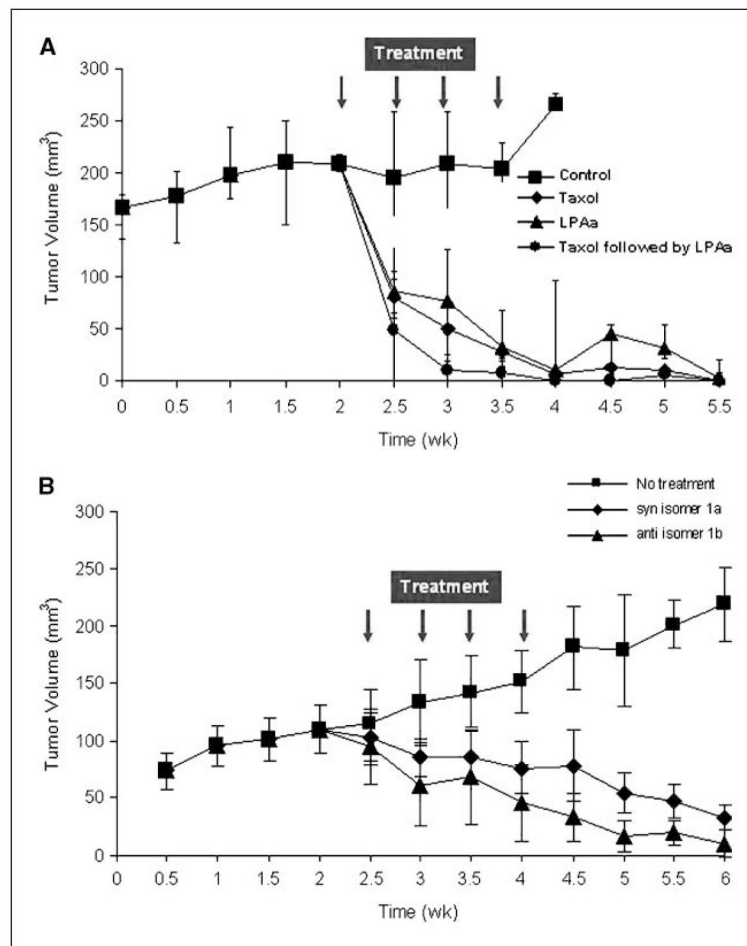


**Figure 2.** Docked complexes of *syn-1a* and *anti-1b* in LPA<sub>1-3</sub> accurately predict pharmacology. *Syn-1a* and *anti-1b* are ball-and-stick models, receptor residues are labeled stick models, and ribbons show the protein backbone (*red*, amino terminus; *blue*, carboxy terminus). *A*, typical view shows the *syn* isomer **1a** in the LPA<sub>2</sub> receptor, with the ligand positioned at the interface between the transmembrane  $\alpha$ -helical segments and the extracellular loops (*top*). *B* and *C*, close views of *syn-1a* and *anti-1b*, respectively, docked in the LPA<sub>1-3</sub> receptors as viewed from the extracellular side. Interactions differing significantly between the complex of each receptor with **1a** and **1b** have green lines.

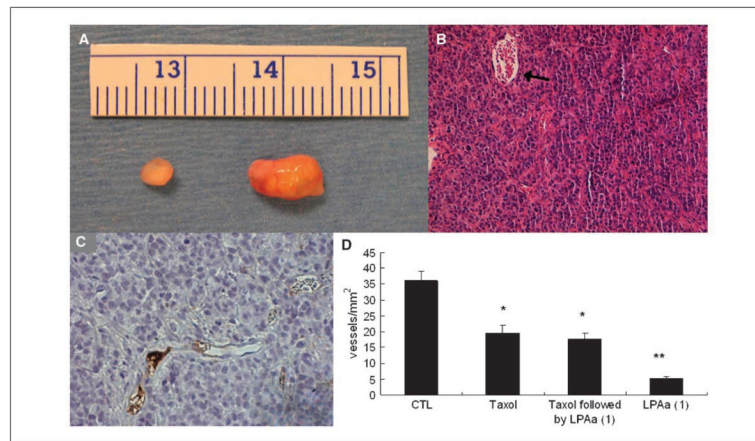


**Figure 3.**

Effect of *syn-1a* and *anti-1b* BrP-LPA diastereomers on MDA-MB-231 cell migration. *A*, confluent MDA-MB-231 cells were scratched and then treated with *syn-1a* or *anti-1b* (10 and 40  $\mu\text{mol/L}$ ) and compared with untreated cells (*CTL*) at 24 h. *B*, quantification for the *anti*-BrP-LPA **1b** and *syn*-BrP-LPA **1a**. Asterisks indicate significant differences from control (*CTL*) at  $P < 0.0005$  (\*) and  $P < 0.0001$  (\*\*) for *syn* isomer **1a** and at  $P < 0.001$  (\*) and  $P < 0.0001$  (\*\*) for *anti*-isomer **1b**.



**Figure 4.** BrP-LPA treatment reduces tumor size in orthotopic breast cancer xenografts. *A*, effect of BrP-LPA **1** treatment (10 mg/kg i.p.) on MDA-MB-231 tumor growth *in vivo*. At 6 wk, all tumor volumes in treated animals were significantly different from the controls but not different from each other ( $P < 0.05$ ). *B*, effect of *syn*-BrP-LPA **1a** and *anti*-BrP-LPA **1b** treatment (3 mg/kg i.p.) on MDA-MB-231 tumor growth. At 6 wk, **1a** and **1b** were statistically different from controls ( $P < 0.05$ ) but not from each other ( $P = 0.16$ ).



**Figure 5.** BrP-LPA decreases tumor size and vascularity. *A*, difference in gross tumor size. H&E staining (*B*) and CD31-specific staining (*C*) show relative angiogenesis. *D*, quantification of newly generated vessels in the tumor samples. The asterisk (\*) indicates that Taxol alone and Taxol then BrP-LPA **1** (mixed isomers) were statistically different from the control ( $P < 0.05$ ) but not different from each other. Treatment with BrP-LPA **1** alone showed significantly lower blood vessel density (\*\*) than the controls ( $P < 0.001$ ) or either Taxol treatment ( $P < 0.05$ ).



Table 1

Pharmacological results with **1**, **1a**, and **1b**

	LPA <sub>1</sub>	LPA <sub>2</sub>	LPA <sub>3</sub>	LPA <sub>4</sub>	LPA <sub>5</sub>	ATX
<b>1</b>	Antagonist IC <sub>50</sub> : 4520 ± 1521 nmol/L K <sub>i</sub> : 805 nmol/L	Antagonist IC <sub>50</sub> : 468 ± 322 nmol/L K <sub>i</sub> : 245 nmol/L	Antagonist 71.3% inhibition at 30 μmol/L (200 nmol/L LPA)	Antagonist 13.9% inhibition at 30 μmol/L (400 nmol/L LPA)	Partial agonist EC <sub>50</sub> : 1282 ± 222 nmol/L E <sub>max</sub> : 54% at 10 μmol/L	102 ± 2.7% inhibition (10 μmol/L)
<b>1a</b>	Antagonist IC <sub>50</sub> : 648 ± 475 nmol/L K <sub>i</sub> : 273 nmol/L	Antagonist IC <sub>50</sub> : 288 ± 103 nmol/L K <sub>i</sub> : 250 nmol/L	Antagonist IC <sub>50</sub> : 4440 ± 1850 nmol/L K <sub>i</sub> : 1830 nmol/L	Antagonist 35.1% Inhibition at 30 μmol/L (400 nmol/L LPA)	Partial agonist NS E <sub>max</sub> : 64% at 10 μmol/L	98.1 ± 2.3% inhibition (10 μmol/L)
<b>1b</b>	Antagonist IC <sub>50</sub> : 2079 ± 1544 nmol/L K <sub>i</sub> : 752 nmol/L	Antagonist IC <sub>50</sub> : 275 ± 133 nmol/L K <sub>i</sub> : 241 nmol/L	Antagonist IC <sub>50</sub> : 2089 ± 1212 nmol/L K <sub>i</sub> : 623 nmol/L	Antagonist 33.1% Inhibition at 30 μmol/L (400 nmol/L LPA)	Antagonist IC <sub>50</sub> : 977 ± 493 nmol/L K <sub>i</sub> : 376 nmol/L	99.4 ± 2.7 % Inhibition (10 μmol/L)

Abbreviation: NS, non-saturated.