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 α-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. [Cancer Res 2009;69(13):5441–9]

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 (PIP3) and lysophosphatidic acid (LPA). In both pathways, down-regulated tumor suppressors and up-regulated oncogenes result in a systemic lysophosphatidic acid (LPA). In both pathways, down-regulated of BrP-LPA was more effective than


tatically important inhibitors of the phosphatidylinositol 3-kinase (1) are present in many advanced cancers. Quite recently, therapeu-

tumor suppressors and up-regulated oncogenes result in a systemic lysophosphatidic acid (LPA). In both pathways, down-regulated

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diastereomeric mixture also inhibited over 98% of ATX activity at 10 μmol/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)butylphosphonate (1b). To a solution of phosphonate 10b (120 mg, 0.18 mmol) in 8 mL EtOAc was added PtO2 (6 mg). The mixture was stirred under 1 atm H2 atmosphere for 20 min, filtered, and concentrated to give anti isomer 1b (87 mg, 100%). [α]D25 = –32.1 (c 0.5, CHCl3); 1H NMR (400 MHz, CDCl3) δ 4.13 (m, 1H), 2.15-2.08 (m, 2H), 1.54 (m, 2H), 1.18 (m, 24H), 0.80 (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, 2H); 13C NMR (101 MHz, CDCl3) δ 174.6, 68.1, 66.7, 40.9, 39.3, 36.6, 34.2, 32.1, 29.8, 29.7, 29.6, 29.5, 29.4, 29.3, 29.5, 22.8, 14.1; 31P NMR (162 MHz, CDCl3) δ 20.21 (1P); MALDI-HRMS [M + Na]+ calcd for C20H40BrO6PNa 509.1638, 511.1621, found 509.1634, 511.1557.

(b) 1(R)-Bromo-3(S)-hydroxy-4-(palmitoyloxy)butylphosphonate (1a) was obtained from 10a in 94% yield analogously, as described for compound 1b. [α]D25 = +3.2 (c 0.5, CHCl3); 1H NMR (400 MHz, CDCl3) δ 4.13 (m, 1H), 2.05-2.00 (m, 2H), 1.94-1.85 (m, 1H), 1.63-1.56 (m, 2H), 1.18 (m, 24H), 0.80 (t, J = 7.2 Hz, 3H); 13C NMR (101 MHz, CDCl3) δ 174.6, 66.7, 66.6, 34.2, 32.1, 29.8, 29.7, 29.6, 29.5, 29.4, 29.3, 25.0, 22.8, 14.1; 31P NMR (162 MHz, CDCl3) δ 19.64 (1P); MALDI-HRMS [M + Na]+ calcd for C20H40BrO6PNa 509.1638, 511.1621, found 509.1639, 511.1605.

Receptor activation. Assays for mobilization of intracellular Ca2+ were performed as described (15, 24), with details in the Supplementary Data. Each test was performed in quadruplicate. EC50, IC50, and Ki 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 LPA1-3. 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 × 108), genetic algorithm search generations (3 × 105), maximum local search iterations (3 × 105), 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-LPA₄, 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), 30 μL of ATX-HA (0.25 μg) in assay buffer [Tris 50 mM, NaCl 140 mM, KCl 5 mM, CaCl₂ 1 mM, MgCl₂ 1 mM (pH 8.0)] was mixed with 25 μL of FS-3 (final, 1 μM) and 25 μL 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 FLExstation 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 ± 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 × 10⁵ 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 μM) 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 Trascel (Glycosan BioSystems) with a final concentration of 5 × 10⁶ cells/mL and the resulting suspension was mixed gently. An aliquot of 200 μL 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 (mm³) = [width (mm)]² × [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 400 magnification, and the data were converted to microvessel density (vessels/mm²), with 1 microscopic field = 0.196 mm². 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 μL 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 ± 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 α-hydroxymethylene phosphonates (Fig. 1B). Aldehyde 4 was prepared in two steps in high yield from acetonide 3 and converted to the diastereomeric mixture of α-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 α-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 α-C in 7b was confirmed by coupling constants and 1H-1H NOESY. Specifically, NOEs were observed between H1 and H3, confirming the syn-1,3-relationship in phosphonate 7b. In 7a, no NOEs were observed between H1 and H3, confirming the anti-1,3 relative stereochemistry. In addition, the acetonide methyls of 7b displayed 13C 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 Ca²⁺ mobilization assay to assess activation or inhibition of LPA₃, LPA₄, LPA₅, and LPA₅ expressed in RH7777 cells and LPA₄ expressed in Chinese hamster ovary (CHO) cells. Table 1 illustrates calcium responses elicited through the response of human LPA₁, LPA₂, LPA₃, LPA₄, and LPA₅ 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 the diastereomeric mixture of phosphonates 5a and 5b.

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

<table>
<thead>
<tr>
<th></th>
<th>LPA₁</th>
<th>LPA₂</th>
<th>LPA₃</th>
<th>LPA₄</th>
<th>LPA₅</th>
<th>ATX</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>4520 ± 1521 nmol/L</td>
<td>468 ± 322 nmol/L</td>
<td>Antagonist 13.9% inhibition at 30 μM/L</td>
<td>Partial agonist 1282 ± 222 nmol/L</td>
<td>102 ± 2.7% inhibition at 10 μM/L</td>
</tr>
<tr>
<td></td>
<td>Kᵢ = 805 nmol/L</td>
<td>Kᵢ = 245 nmol/L</td>
<td>(400 nmol/L LPA)</td>
<td>Eₘₐₓ 54% at 10 μM/L</td>
<td>Eₘₐₓ 64% at 10 μM/L</td>
<td>98.1 ± 2.3% inhibition at 10 μM/L</td>
</tr>
<tr>
<td>1a</td>
<td>648 ± 475 nmol/L</td>
<td>288 ± 103 nmol/L</td>
<td>Antagonist 35.1% Inhibition at 30 μM/L</td>
<td>Partial agonist NS</td>
<td>99.4 ± 2.7% inhibition at 10 μM/L</td>
<td></td>
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<tr>
<td>1b</td>
<td>2079 ± 1544 nmol/L</td>
<td>275 ± 133 nmol/L</td>
<td>Antagonist 33.1% Inhibition at 30 μM/L</td>
<td>Partial agonist 977 nmol/L</td>
<td>Inhibition</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Kᵢ = 752 nmol/L</td>
<td>Kᵢ = 241 nmol/L</td>
<td>(400 nmol/L LPA)</td>
<td>Kᵢ = 376 nmol/L</td>
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**Abbreviation:** NS, non-saturated.
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 LPA1-4 submicromolar potency toward LPA2, modest inhibition of LPA activation of LPA4, and weak partial agonism for the transiently transfected LPA5. The syn-BrP-LPA 1a was a pan-antagonist for the edg family GPCRs, showing Ki values of 273, 250, and 1830 nmol/L for LPA1, LPA2, and LPA3, respectively. For the purinergic family GPCRs, syn-BrP-LPA 1a was an antagonist for LPA4 but a weak agonist for LPA5. 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 Ki values of 752, 241, and 623 nmol/L for LPA1, LPA2, and LPA3, respectively. In addition, anti-BrP-LPA 1b was an antagonist for the two purinergic family GPCRs, with partial antagonism of LPA on LPA4 and a Ki value of 376 nmol/L for LPA5.

**Molecular modeling of diastereoisomers with LPA1, LPA2, and LPA3.** Molecular models of the complexes formed by syn-BrP-LPA 1a and anti-BrP-LPA 1b with LPA1-3 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 NH2 terminus, third transmembrane domain, and seventh transmembrane domain in LPA1-3 (Fig. 2). The basic NH2 terminal residues showed variable position and type, with LPA1 interacting via R36, LPA2 interacting via H91, and LPA3 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 SIP recognition by the EDG family SIP receptors (36, 37). The interacting residue in the seventh transmembrane domain of LPA1 and LPA2 is K7.36, whereas LPA3 uses R7.36. Moreover, K7.35 in LPA5, not R7.36, is essential for LPA recognition, although this lysine is replaced by an acidic residue in LPA1 and LPA2 (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 μmol/L and compared with the ATX inhibitory effects of 10 μmol/L LPA (18:1) and 10 μmol/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 Km value of 6.3 μmol/L (29). Preliminary results showed that each analogue inhibited >98% of ATX at 10 μmol/L. A clear dose-response effect on inhibition was observed for both syn-BrP-LPA 1a (IC50 = 165 nmol/L) and anti-BrP-LPA 1b (IC50 = 22 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 LPA1 > LPA2 >>
LPA₃ and LPA₁ 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 μmol/L inhibited the MDA-MB-231 cell migration (Fig. 3B). Migration of MB-231 cells was decreased by 57% (P < 0.05) by 40 μmol/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

![Figure 3.](image-url). 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 μmol/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.
injected cell suspension was reduced to 100 μL 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 that 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 LPA1-3 were performed computationally without knowledge of the pharmacologic outcomes. The predictions from the models were gratifyingly congruent with the relative antagonist activity at LPA1-3, 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 LPA2 are essentially identical, consistent with equivalent potencies. Whereas both compounds show similar phosphate basic residue distances in LPA3, 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_{1-5}. This represents a proof of concept that compounds capable of inhibiting both the edg family of LPA receptors (LPA_{1-3}) and the purinergic family of LPA receptors represented by LPA_{4} and LPA_{5} 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 motogenic 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 μmol/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.

**Theoretical potential of ATX inhibitors and LPA antagonists in cancer treatment.** LPA GPCRs and ATX present two promising, druggable, yet underexploited targets for cancer therapy (5, 6). ATX, a potent motogen 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 cyclophosphatic acid (ccPA) showed that these compounds were selective inhibitors of ATX and lacked agonist activity for LPA1a,1b,3 (38). Importantly, ccPA inhibited cancer cell invasion in vivo 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.

**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 Echelon Biosciences. The other authors declared no potential conflicts of interest.

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LPA Signaling Antagonist Inhibits Metastatic Phenotype

Dual Activity Lysophosphatidic Acid Receptor Pan-Antagonist/Autotaxin Inhibitor Reduces Breast Cancer Cell Migration \textit{In vitro} and Causes Tumor Regression \textit{In vivo}

Honglu Zhang, Xiaoyu Xu, Joanna Gajewiak, et al.