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Identification of Non-Lipid LPA₃ Antagonists by Virtual Screening

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Abstract

In the present study, we utilized virtual screening to identify LPA₃ antagonists. We have developed a three point structure-based pharmacophore model based on known LPA₃ antagonists. This model was used to mine the NCI database. Docking, pharmacophore development, and database mining produced new, non-lipid leads. Experimental testing of seven computationally-selected pharmacophore hits produced one potentiator and three antagonists, one of which displays both LPA₃ selectivity and nanomolar potency. Similarity searching in the ChemBridge database using the most promising lead as the search target produced five additional LPA₃ antagonists and a potent dual LPA_{1&2} antagonist.

Keywords

lysophosphatidic acid; GPCR; database mining; pharmacophore

Introduction

Lysophosphatidic acid (LPA) is a phospholipid mediator that elicits a host of biological effects including cell proliferation, survival, motility and differentiation.^{1,2} LPA evokes many of these responses extracellularly through G protein-coupled receptors (GPCR) that belong either to the endothelial differentiation gene family: EDG2/LPA₁, EDG4/LPA₂, EDG7/LPA₃,³ or to a subcluster in the purinergic GPCR family p2y9/LPA₄,⁴ GPR92/LPA₅,⁵ and GPR87/LPA₆.⁶ LPA receptors are implicated in cardiovascular disease and atherosclerosis through their action on platelets, leukocytes, and the different cell types of the arterial wall.^{7,8} LPA has been shown to regulate cancer cell invasion, metastasis, and resistance to both chemotherapeutics and radiation.^{9,10} Due to these many therapeutically relevant effects of LPA, highly selective receptor antagonists are important, but not yet available. The specific role of the LPA receptors in these diseases is difficult to assess because many cells express combinations of LPA receptors.^{1,2} Subtype-specific antagonists could also serve as important tools to elucidate the pathological and physiological roles elicited by a particular LPA subtype.

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LPA₃ shows a limited expression pattern in the testes, prostate, pancreas, heart, lung, and ovary^{11–13}, unlike LPA₁ or LPA₂, which are expressed in almost all tissues.¹¹ LPA and the LPA₃ receptor in particular is important in embryo implantation and uterine motility.¹⁴ This restricted expression and the distinct biological functions make LPA₃ an attractive drug target for gynecological and reproductive diseases. Identification of selective LPA₃ antagonists in particular has the potential to contribute to studies of the role of LPA in cardiovascular disease and reproduction.

Several LPA receptor antagonists (Figure 1) have been reported to date but all, with the exception of the non-selective Ki16425¹⁵, are lipids which do not satisfy Lipinsky's rule of five.¹⁶ No single receptor-selective antagonists for any of the six LPA receptors have been reported. Ohta et al. showed that Ki16425 is a non-selective LPA_{1/2/3} antagonist.¹⁵ Ki16425 is an isoxazole derivative that has greater potency for LPA₃ than LPA₁ and LPA₂.¹⁵ Fatty alcohol phosphates (FAP) with 12- and 14-carbon alkyl chains and the short-chain phosphatidic acid analogs dioctylglycerol phosphate (DGP) and dioctylglycerol thiophosphate (DGTP), are selective for LPA₁ and LPA₃ over LPA₂.^{17,18} DGP and DGTP have IC₅₀ values of 143 and 184 nM, respectively, for the LPA₃ receptor and their IC₅₀ values at the LPA₁ receptor are only 2- and 10-fold higher than for LPA₃. Although several additional LPA₃ antagonists have been reported,^{17–20} (Figure 1) their structures are closely related to these lipid-like antagonists. Ki16425, DGP, and DGTP, therefore, represent the available diversity that defines the spatial arrangements of functional groups necessary for LPA₃ antagonist activity (the pharmacophore) and can potentially assist in identification of other, structurally unrelated, compounds.

The dual action of the presently known antagonists at both LPA₃ and LPA₁ suggests that information regarding the active conformation at the LPA₃ receptor is critical for successful pharmacophore development. Since no crystal structure of the LPA₃ receptor is currently available, such information at the present time can only come from modeling studies. We have modeled the LPA_{1–3} receptor structures and complexes with both agonists and antagonists.^{21–23} Extensive computationally-guided mutagenesis studies have confirmed key residues responsible for agonist binding.²⁴ Residues experimentally confirmed to be important for LPA recognition in LPA₃ using the nomenclature of Ballesteros and Weinstein²⁵ are Arg 3.28 and Glu 3.29, which are conserved in the LPA_{1–3} receptors, along with Lys 7.35 and Arg 5.38.²¹

In the present study pharmacophore development, database mining, and docking have been applied to identify subtype-specific, non-lipid LPA₃ antagonists. Based on the previous computational models of the LPA receptors^{21,23,26} along with SAR data for known LPA antagonists,^{15,17–20,27,28} we developed a structure-based pharmacophore. We pursued a structure-based approach to account for key interactions of the ligands with the LPA₃ receptor. This allowed definition of a pharmacophore specific to LPA₃ antagonism using the currently known antagonists even though they display dual activity at both LPA₁ and LPA₃. The resultant pharmacophore was used to search databases for potential antagonist leads. Rigid docking afforded us the capability to rapidly screen and further refine the hits generated from our database searching. Pharmacophore searching of the NCI database²⁹ and further hitlist refinement identified seven potential LPA₃ antagonists. Pharmacological assays were used to establish receptor selectivity. Pharmacological screening confirmed one LPA₃ potentiator and four LPA₃ antagonists with submicromolar K_i, one of which displays both LPA₃ selectivity and nanomolar potency. Single reference similarity searching in the ChemBridge database yielded five more LPA₃ receptor antagonists, and a full LPA_{1&2} antagonist. Here we report the first antagonist with receptor selectivity for LPA₃ over LPA_{1/2/4/5}.

Results

Docking simulations using the inactive LPA₃ model were performed with the three known LPA₃ antagonists, DGP, DGTP, and Ki16425. These studies provided insight into interactions that may be important for antagonist activity by comparing docking simulations of each antagonist in the LPA₃ receptor. Key residues predicted to interact with the ligands were R3.28, R7.36, K95, K7.35, and H5 (Figure 2). In particular, docking studies suggested the anionic group of all three ligands ion paired with R7.36, K95, and R3.28 (Table 1). These data and prior studies suggest that the antagonists and agonists share interactions with R3.28 and R7.36.^{21,23} These electrostatic interactions were deemed to be important for developing competitive antagonists.

A structure based pharmacophore was generated from the superposition of these antagonists. The model takes into account interactions within 4.5 Å of the known antagonists, based on the expectation that these are critical for antagonistic activity. The LPA₃ antagonist pharmacophore is a three-point pharmacophore model consisting of an anionic group and two hydrophobic regions, shown in figure 3. The anionic group is spaced 8–14 Å and 12–16 Å from the two hydrophobic regions. Hydrophobic regions are spaced 7–12 Å apart.

The pharmacophore was used to mine the National Cancer Institute (NCI) Developmental Therapeutics Chemical Database. A search of this NCI database with various anionic functional groups (Table 2) identified 1098 hits. One of the hits included the known LPA₃ antagonist, dodecyl phosphate (FAP 12:0)¹⁸. Many of the hits, including mesuprine, methotrexate, and N6-benzyladenosine-5'-phosphate (NBAP, Figure 4) are structurally distinct from known antagonists, offering diversity in new candidate structures.

In silico screening was used to reduce the pharmacophore hits to the most promising compounds for pharmacological assay. Visual assessment prior to rigid docking was used to evaluate whether the compound was too large in size. This assessment slightly reduced the 1098 compound hit list.

One hit, NBAP, when tested experimentally did not show either an antagonist or agonist response, but acted as a potentiator when co-administered with LPA (Figure 5A and Table 4). Further analysis revealed that NBAP matched the LPA₃ agonist as well as antagonist pharmacophore (Figure 5B). This result necessitated that LPA₃ antagonist pharmacophore hits matching the pharmacophores for other LPA activities be eliminated to promote identification of more selective leads. LPA₁ antagonist and LPA₃ agonist pharmacophores were available for this additional filtering step (Table 3). Comparison to these pharmacophores produced a refined hitlist of 212 compounds.

The Developmental Therapeutics Program (DTP) at the National Cancer Institute provided samples of seven compounds for screening including: NSC47091, NSC161613, NSC18749, NSC1741, NSC48776, NSC168199 and NSC343949. Assay data for the six non-fluorescent compounds (structures in Figure 6) provided by the DTP are reported in Table 4. Two compounds, NSC343949 and NSC48776, had no antagonist or agonist effect on LPA₁₋₃. NSC18749 was shown to be a weak agonist of LPA₁₋₃. NSC47091, NSC1741, and NSC161613, showed the predicted antagonist effect. NSC161613 was identified as being a selective partial antagonist of LPA₃ (IC₅₀ 24 nM) over LPA_{1/2/4/5}. NSC47091 was dual antagonist for both LPA₂ and LPA₃ (IC₅₀ 355 nM and 30 nM, respectively). NSC1741 was a pan-antagonist for LPA₁, LPA₂ and LPA₃ in the micromolar concentration range.

Three of the six non-fluorescent compounds provided by NCI, NSC47091, NSC1741, and NSC161613, were LPA₃ antagonists, as predicted by the *in silico* screening experiments. Docking simulations revealed that these leads exhibit several ionic interactions with LPA₃

residues that may be important for antagonist activity including K95, R3.28, and R7.36 (Table 5). Figure 7 shows the geometric fit of these three docked antagonists inside the LPA₃ pharmacophore. All three antagonists place anionic functional groups within or near the anionic pharmacophore sphere, but do not occupy both hydrophobic points when docked into the receptor. This failure to occupy the third point may explain the partial, rather than full, antagonism observed. All active compounds were predicted to have at least four ionic/polar interactions. In contrast, the inactive compounds were predicted to have three or fewer ionic/polar interactions.

NSC47091 acted as an antagonist at LPA₂ as well as LPA₃. The pharmacological profile of NSC47091 can be explained by two amino acid differences between LPA₁, LPA₂ and LPA₃. First, docking predicts that the ionic interaction of the carboxylate with K7.35 in LPA₃ cannot occur in LPA₁ or LPA₂, which contain a glutamate at the corresponding site. Second, antagonist activity still occurs in LPA₂ due to an ionic interaction with H2.63, which corresponds to asparagine in the corresponding sites of LPA₁ and LPA₃.

NSC161613 was computationally and experimentally selective for LPA₃. The modeling of NSC161613 in LPA₃ showed several amino acids that might contribute to its selectivity (Figure 8). First, an ionic interaction was observed between NSC161613 and H5. H5 corresponds to F25 in LPA₁ and Y8 in LPA₂. Second, R267 is predicted to interact with the nitro group of this compound. This interaction was lacking in both LPA₁ and LPA₂ where the R267 residue corresponds to proline and glutamate, respectively. Finally, K95 in LPA₃ is predicted to interact with the two carboxylate groups suggesting a stabilizing ionic interaction of the complex. This interaction is not present in LPA₂ where the corresponding residue is a proline.

Similarity searching using the online search tool www.hit2lead.com (ChemBridge) using NSC47091, the most efficacious antagonist, as the search target yielded 546 hits. Visual assessment ultimately reduced this number to 12 compounds that were virtually screened. Five compounds (Figure 9) exhibiting at least four ionic/polar interactions with the LPA₃ residues identified in Table 5 were purchased and experimentally screened. Four out of the five were found to be LPA₃ antagonists with varying degrees of inhibitory effect at the other two LPA receptors (Table 6), whereas compound H2L 5105099 was an LPA_{1&2} antagonist. Absence of the acid on the ring in H2L 5105099 compared to NSC47091 appears to be responsible for LPA₁ antagonism and the lack of effect at LPA₃ as this compound was a potent full antagonist for both LPA_{1&2}, with *K_i* values of 50 and 21 nM, respectively. Although H2L 5186303 displayed full antagonism of LPA₁₋₃, it was more than 40 and 1800 fold more selective for LPA₂ than either LPA₃ or LPA₁.

Discussion

In the present study we describe the usefulness of a structure-based pharmacophore for identifying new structural classes of LPA₃ antagonists. Previous LPA₃ antagonist discoveries utilized either SAR studies of LPA analogs or high-throughput screening and produced predominantly lipid antagonist classes.^{15,20,30} SAR studies used an anionic head group, linker atom, and a bulky alkyl group as design features.^{20,30} Our study used docking to generate a pharmacophore hypothesis which took into account the predicted interactions of the ligand and the receptor.

The NCI database is the largest freely available three-dimensional chemical database. It consists of over 250,000 compounds of which 200,000 are not found in any other database.³¹ Conformations for the 250,000 compounds in the NCI database were generated using Catalyst. Up to 25 conformers have been generated for each compound yielding over 2 million conformers for the 250,000 compounds in the database.³² This allows rapid searches for rigid

docking candidates. Admittedly, 25 conformations is not an exhaustive set for flexible molecules. Incomplete representation of the conformational space of flexible molecules will result in failure to identify some flexible molecules as hits that are able to adopt conformations matching our pharmacophore. This result is acceptable as highly flexible molecules are likely to exhibit poor selectivity against the LPA receptor subtypes.

We used this database to begin identifying novel leads as potential antagonists of LPA₃. The database search utilized both geometric and physicochemical query terms. A log P range between two and negative two was used to reduce the number of lipid hits.³³ Virtual screening using rigid docking allowed us to rapidly screen the hits for their steric and electrostatic complementarity to the receptor. Compounds were then prioritized for pharmacological assay. This approach provided a good approximation of the bioactive conformation for LPA₃ antagonists. In another study, an EDG receptor pharmacophore for SIP₃ antagonism was derived without receptor structural input, resulting in 307 possible pharmacophores.³⁴ Application of a filtering method reduced this set to seven pharmacophore hypotheses, which produced 3 compounds active at low micromolar concentration out of 36 compounds experimentally screened.

Using hits uniquely fit to our single pharmacophore hypothesis of LPA₃ antagonism and not matching the LPA₁ antagonist or LPA₃ agonist pharmacophores, we were able to find compounds with no detectable antagonism at LPA₁ that were both non-lipid and more potent than previously described LPA₃ antagonists after screening only seven compounds. The most efficacious compound, NSC47091, was used in similarity searching. Analogs identified by similarity searching can be used to better outline pharmacophore features needed. Five non-lipid LPA₃ antagonists and a full antagonist selective for LPA_{1&2} were identified.

Using a three-point pharmacophore, we were able to identify three non-lipid LPA₃ receptor antagonists. NSC47091, NSC1741, and NSC161613 are all distinctly different from the antagonists used for the pharmacophore development and previously reported antagonists. NSC47091 and NSC161613 showed IC₅₀ values of 30 nM and 24 nM, respectively, competing against 200 nM LPA 18:1 at LPA₃. Both compounds are more potent than previously reported antagonists, which showed IC₅₀ values ranging from 175 to 9260 nM competing against 10 μ M LPA.^{18,20,27,30} In our hands, three previously published antagonists, DGPP, Ki16425, and VPC12249 show LPA₃ K_i values of 202 nM, 148 nM, and 588 nM, respectively. NSC47091 and NSC161613 showed no antagonist activity for LPA₁, making them unique among currently known LPA₃ antagonists, which all demonstrate some antagonism of LPA₁. NSC47091 is the first reported dual antagonist of LPA₂ and LPA₃. NSC161613 was notably the first reported completely selective antagonist for LPA₃ over LPA_{1/2/4/5}.

One unexpected finding from this research was the novel LPA₃ potentiator, NBAP (Figure 4). This is the first report of an allosteric regulator of LPA₃. While it is possible that the allosteric site is found within a single LPA₃ receptor monomer, the excellent fit of NBAP onto docked LPA₃ receptor agonists (Figure 5B) suggests that the allosteric site might be an orthosteric site (the site responsible for agonist recognition) in the second monomer in a dimer, as reviewed by Springael, *et al.*³⁵ This possibility is particularly compelling in light of the finding that LPA₃ formed homodimers in a lacZ complementation assay.³⁵

Single reference similarity searching was done in an attempt to find analogs of an antagonist identified our pharmacophore search. NSC47091 was chosen as the reference compound because it had the greatest inhibition efficacy (I_{max}=82%) in comparison to the other two compounds identified (I_{max} < 70%). Modeling of NSC47091 and LPA₃ suggested that the anionic group on the ring was important for antagonist activity, due an ionic interaction with K7.35 in the receptor. By exploring the effects of the removal of the anionic group on the ring,

we found that the anionic group was needed for LPA₃ selectivity. Figure 10A shows an overlay of NSC47091 and H2L5105099 indicating that both compounds dock in similar positions. Figure 10B shows that the most potent nonselective antagonist found (H2L518603, LPA₃ Ki=310 nM) in similarity searching places an electronegative group in a similar position as the carboxylate group of NSC47091. This substitution is sufficient for LPA₃ antagonism, but does not lead to selectivity. These considerations suggest that in order to eliminate the LPA₁ antagonist effect, a central anionic group is required.

Conclusion

In summary, we have identified nine antagonists of LPA-elicited calcium mobilization using RH7777 cells individually expressing each of the EDG-family LPA receptors. One of these, NSC161613, is the first reported selective LPA₃ receptor antagonist. It is a potent antagonist selective for the LPA₃ receptor with no detectable activity at LPA_{1/2/4/5}. Another compound, H2L5186303, shows a 40- to 1800-fold selectivity for the LPA₂ receptor over LPA_{1/3} with a 7.2 nM Ki. The five antagonists found by similarity searching provided data to refine the LPA₃ antagonist pharmacophore to include a fourth point promoting LPA₃ selectivity. This could not have been previously recognized based on the previous known non-selective LPA₃ antagonists. The combination of our pharmacophore model and *in silico* screening strategy functions as an efficient tool for identifying novel leads for the LPA₃ receptor. Efforts are ongoing to identify additional antagonists and to optimize leads using other computational methods.

Methods

Pharmacophore Design

The pharmacophore was developed from the structure-based superposition of three known LPA₃ antagonists, the lipid-like DGP, DGTP, and non-lipid Ki16425. The three known antagonists were built in the MOE³⁶ molecular modeling software package. Each of the antagonists was modeled in the ionization state expected at pH 7 and partial charges were assigned using MMFF94³⁷. The antagonists were then individually flexibly docked using Autodock 3.0³⁸ inside the inactive LPA₃ receptor model.²³ The inactive LPA₃ receptor model, as previously described²³ is a homology model based on a crystal structure of the dark-adapted bovine rhodopsin³⁹. Autodock 3.0 was used to identify the receptor-bound conformations of each antagonist. Default parameters of Autodock 3.0 were used with the following exceptions: energy evaluations(9×10^{10}), genetic algorithm search generations(3×10^4), maximum local search iterations(3×10^3), and runs (15). The docking box dimensions were $21.375 \text{ \AA} \times 21.375 \text{ \AA} \times 34.875 \text{ \AA}$, with the long dimension following a line from the top of TM1 to TM4. The box was centered to include residues R276, K275, I173, L86, R105, W102, C171, N172, N89, and T90. Fifteen complexes of each antagonist were generated. The lowest docked energy complex of each antagonist was then minimized using the MMFF94³⁷ forcefield. In MOE³⁶ the individual complexes were superimposed on each other and residues within $4.5 \text{ \AA}$ of the ligands were analyzed for common ligand interactions amongst the complexes. A three point pharmacophore hypothesis was derived consisting of two hydrophobic regions and one anionic region based on common volume occupied and receptor interactions. The distances between these points were measured to mine databases for compounds matching these dimensions.

Mining the NCI database

The distances derived from the pharmacophore hypothesis were used to search the NCI database. The database query consisted of two filters: 1) The antagonist pharmacophore hypothesis and 2) Log P range between -2 and 2 to minimize the number of lipids given as

hits. Multiple anionic groups were utilized including phosphate, carboxylate, sulfate, and sulfonamide.

Compound selection

The hits generated from the LPA₃ antagonist pharmacophore were further filtered by crossing these hits with the hits generated from the LPA₁ antagonist and LPA₃ agonist pharmacophores to be reported elsewhere. The conformation of each hit matching the LPA₃ antagonist pharmacophore was downloaded as a SYBYL mol2⁴⁰ format file from the NCI database.

The hits were then rigidly docked in the inactive LPA₃ receptor model using Autodock 3.0. Compounds were docked as before with the exception that 5000 generations instead of 30000 were sufficient to obtain convergence for rigid ligands. The lowest energy receptor: ligand complex of each hit was then analyzed for intermolecular interactions within 4.5 Å.

A qualitative assessment comparing the docked hits of the NCI database to those used for pharmacophore development was done to select compounds for experimental testing. Priority for experimental evaluation was assigned by identifying the compounds that exhibited interactions at amino acid residues seen in complexes of the known antagonists, particularly those also required for agonist binding, and additional interactions at other amino acid residues.

Similarity Searching

Similarity searching was performed using the ChemBridge online database, www.hit2lead.com. Leads experimentally confirmed as antagonists selected using pharmacophore screening were used as search target compounds. The similarity threshold was set at 60%. Compounds were visually assessed to determine how closely the structure closely mimicked the reference compound. Filtered hits were then flexibly docked into the inactive LPA_{2&3} receptor models. Ligand/receptor interactions were assessed and compounds were selected for biological screening using the criteria defined in the Compound Selection subsection.

Synthesis

All reagents were purchased from Sigma-Aldrich Chemical Company and Toronto Research Chemicals and were of the highest available purity. The purity of all reagents was verified by ¹H NMR. All ¹H NMR spectra were recorded on a JEOL 270-MHz spectrometer. Chemical shifts are expressed in parts per million (PPM) downfield from an internal tetramethylsilane standard. Electrospray mass spectrometric data [M-H]⁻ was collected on a ThermoFinnigan LCQ Advantage LC-MS in the negative ion mode by direct infusion of 20 ng/μl methanol solutions.

Synthesis of isopropylidene protected N-benzyladenosine (1). To a magnetically stirred solution of N-benzyladenosine (500 mg, 1.40 mmol) in anhydrous acetone (150 mL) was added *p*-toluenesulfonic acid monohydrate (2.4 g, 14 mmol). The resultant pale yellow solution was maintained in an anhydrous, room temperature environment. At 15 minute intervals, an aliquot of the acidic reaction mixture was removed, neutralized with a small excess of 0.5 M sodium bicarbonate solution, and analyzed via thin-layer chromatography (TLC, solvent system: 85% aqueous ethanol solution). After 45 minutes, the conversion of N-benzyl adenosine (R_f = 0.65) to the isopropylidene derivative (R_f = 0.82) was complete. The pale yellow solution was subsequently mixed with 175 mL of 0.5 M sodium bicarbonate solution and dried *in vacuo*, yielding 0.495 g of a white solid (89% yield). ¹H NMR (isopropylidene environments are in bold): **1.33 (s, 3H)**, **1.52 (s, 3H)**, 3.53 (s, 1H), 3.42 (s, 1H), 4.11 (d, 1H), 4.14 (d, 1H), 4.18 (d, 1H), 4.22 (d, 1H), 4.71 (s, 2H), 4.86 (d, 1H), 5.36 (s, 1H), 6.14 (d, 1H), 7.27 (m, 5H), 7.41 (s, 1H), 8.23 (s, 1H), 8.46 (s, 1H).

Synthesis of protected N-benzyladenosine phosphate (2). To a stirred solution of 1 (495 mg, 1.25 mmol) in 30 mL of anhydrous dichloromethane was added di-*tert*-butyl-N,N-diisopropylphosphoramidite (750 mg, 2 mmol) and ¹H-tetrazole (275 mg, 4 mmol). The reaction mixture was allowed to stir for 1h under an inert nitrogen environment at room temperature. The reaction mixture was then cooled to 0°C and 0.3 mL of 30% hydrogen peroxide was added, and the resulting solution was stirred for 1h. The mixture was diluted with 50 mL of dichloromethane, washed with 10% sodium metabisulfite (2 × 25 mL), saturated sodium bicarbonate (2 × 25 mL), water (2 × 25 mL), and brine (25 mL). The organic layer was washed over anhydrous magnesium sulfate, filtered and concentrated *in vacuo* to yield a crude oil (313 mg, 53% yield). The resulting crude product was purified by column chromatography (60% hexane 40% ethyl acetate solvent system) to elute the desired protected phosphate 2 (212 mg, 36% yield from compd 1) as a clear oil. ¹H NMR (t-butyl resonance is in bold): **1.27 (s, 18H)**, 1.33 (s, 3H), 1.52 (s, 3H), 3.53 (s, 1H), 3.42 (s, 1H), 4.11 (d, 1H), 4.14 (d, 1H), 4.18 (d, 1H), 4.22 (d, 1H), 4.71 (s, 2H), 4.86 (d, 1H), 6.14 (d, 1H), 7.27 (m, 5H), 7.41 (s, 1H), 8.23 (s, 1H), 8.46 (s, 1H).

Synthesis of N-benzyladenosine phosphate (3). To a stirred solution of 2 (212 mg, 0.36 mmol) in 50 mL of anhydrous dichloromethane was added trifluoroacetic acid (0.6 mL, 8.7 mmol) and deionized water (36 µL, 2 mmol). The resulting mixture was allowed to stir at room temperature for 5h and reaction completion was verified via TLC. Solvents were removed *in vacuo* and the residue was washed with dichloromethane (2 × 40 mL). Concentration *in vacuo* yielded 3 as a colorless oil (103 mg, 61% yield from 2). ¹H NMR 3.53 (s, 1H), 3.42 (s, 1H), 4.11 (d, 1H), 4.14 (d, 1H), 4.18 (d, 1H), 4.22 (d, 1H), 4.71 (s, 2H), 4.86 (d, 1H), 6.14 (d, 1H), 7.27 (m, 5H), 7.41 (s, 1H), 8.23 (s, 1H), 8.46 (s, 1H). MS: [M-H]⁻ at m/z 476

Measurement of intracellular Ca²⁺ mobilization

The rat hepatoma (RH7777) cell lines individually expressing either LPA₁, LPA₂, LPA₃ or LPA₅ receptors and the Chinese hamster ovary (CHO) cell line expressing LPA₄ were analyzed to examine agonism and antagonism. Wild type RH7777 cells do not respond to LPA with changes in [Ca²⁺]_i. CHO cells stably expressing either vector or LPA₄ were a kind gift from Dr. Shimizu (University of Tokyo, Tokyo, Japan).

Stable transformants of LPA_{1/2/3} receptors: RH7777 cells stably expressing each receptor were plated onto poly-L-lysine (PLL, 0.1 mg/ml)-coated black-wall clear-bottom 96- well plates (Corning Incorporated Life Sciences, Acton, MA) at a density of 5 × 10⁴ cells/well and cultured overnight. The following day, the culture medium was replaced with modified Krebs buffer (120 mM NaCl, 5 mM KCl, 0.62 mM MgSO₄, 1.8 mM CaCl₂, 10 mM HEPES, 6 mM glucose, pH 7.4), and the cells were serum starved for 6 h. Subsequently, cells were loaded with Fura-2 AM (Invitrogen, Carlsbad, CA) for 35 min in modified Krebs buffer containing 2% (v/v) pluronic acid.

Stable transformants of LPA₄: CHO cells stably expressing either vector or LPA₄ were plated onto non-coated 96 well plates at a density of 4 × 10⁴ cells/ well and cultured overnight. The following day, cells were loaded with Fura-2 AM for 1 h in modified Krebs buffer containing 2% (v/v) pluronic acid and 2.5mM probenecid.

Transient transfection of LPA₅: RH7777 cells in 10 cm dish at a density of 2 × 10⁶ were transfected with 2 µg of plasmid DNA with Effectene (Qiagen, Valencia, CA) according to the manufacturer's instructions for 24 h, then replated onto PLL-coated 96- well plates at a density of 5 × 10⁴ cells/well and cultured overnight. The following day, the culture medium was replaced with modified Krebs buffer, and the cells were serum starved for 4 h. Cells were loaded with Fura-2 AM for 30 min in modified Krebs buffer containing 2% (v/v) pluronic acid.

After incubating the cells with Fura-2 AM, the cells were rinsed with Krebs buffer and changes in the intracellular Ca^{2+} concentration were monitored by determining the ratio of emitted light intensities at 520 nm in response to excitation at 340 and 380 nm using a FLEXstation II (Molecular Devices, Sunnyvale, CA). Each well was monitored for 80–120s. For testing agonist activity of the compounds, the test compounds were added automatically after 15 s of baseline measurement. To determine antagonist properties, varying concentrations of the compounds were mixed with constant concentration of LPA and responses were monitored. Each test was performed in quadruplicate. CHO cells endogenously express LPA_1 , therefore, to access the effect of LPA_4 in CHO cells, the response in vector-transfected cells was subtracted from the response in LPA_4 -transfected cells.

One hit, NSC168199, showed strong fluorescence that interfered with the Ca^{2+} assay. Fluorescence of the compounds was determined by dissolving 10 μM of each compound into 100 μl Krebs buffer per well and measuring autofluorescence to excitation at 340 and 380 nm using a FLEXstation II (Molecular Devices, Sunnyvale, CA). NSC168199 was excluded from further testing due to autofluorescence.

Statistical analysis

Significant difference was determined by the Student's test at a P value of 0.05. IC_{50} values were calculated by fitting a sigmoid function to data points by using the nonlinear curve-fitting feature of KaleidaGraph (Synergy Software, Essex Junction, VT).

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Fig. 1.
LPA receptor antagonist structures.

Fig. 2. Models of LPA₃ receptor complexes with antagonists (Ki16425, DGTP, and DGP). Protein shown as ribbon with Ki16425 as a ball and stick model in Panel A. Panels B(DGP), C(DGTP), and D(Ki16425), show antagonist(ball and stick) interactions with key amino acid residues (sticks) from a common perspective.

Fig. 3. Superimposition of docked LPA₃ antagonists DGTP, DGP, and Ki16425 used to develop the LPA₃ antagonist pharmacophore. The pharmacophore features in the 3-point pharmacophore are the two hydrophobic points colored green and one anionic group colored in red. The distances among the three points are 12–16 Å and 8–14 Å from the anionic to the two hydrophobic points, and 7–12 Å between hydrophobic points.

Fig. 4.

Representative non-lipid pharmacophore matches identified in NCI database search.

Fig. 5. NBAP potentiates LPA action at LPA₃. Panel A. Intracellular Ca^{2+} transients (mean $\pm$ SD) were measured in response to the application of increasing concentrations of LPA 18:1 alone (filled squares), NBAP alone (filled circles), or NBAP mixed with 200 nM LPA 18:1 (filled triangles). 100% represents the maximal Ca^{2+} mobilization elicited by LPA 18:1. Panel B. Comparison of NBAP with docked LPA₃ agonists.²⁴ LPA₃ agonists are colored cyan. NBAP was flexibly aligned onto the fixed agonists showing close geometric position of anionic groups, and incomplete volume occupancy by NBAP of the bottom of the agonist binding site. The pink circle shows all phosphate groups in the same position.

Fig. 6.
Compounds provided by DTP for experimental screening.

Fig. 7. Confirmed antagonists identified in pharmacophore searches of the NCI database, NSC161613 (A), NSC47091(B), and NSC1741(C) shown superposed on the LPA₃ antagonist pharmacophore. The three antagonists used for pharmacophore development are shown in purple along with the anionic and hydrophobic pharmacophore points in red and green, respectively.

Fig. 8. Key interactions observed in the NSC161613(ball and stick) complex with LPA₃(selected residues labeled and shown as stick models).

Fig. 9.
Similarity matches based on NSC47091 purchased from Hit2lead for experimental screening.

Fig. 10. Superimposition of docked LPA₃ antagonist structures. Panel A. Superimposition of NSC47091 (Ball & Stick) and H2L5105099 (pink). Lack of LPA₃ activity is attributed to missing electronegative region in H2L5105099. Panel B. Overlay of NSC47091 (Ball & Stick, blue), H2L5186303 (Ball & Stick, purple), and H2L5105099 (Stick, pink). Highlighted in the overlay is the electronegative core required for antagonist activity.

Table 1

Interaction distances (Å) between published antagonists and selected LPA₃ receptor residues. Interactions with distances > 4.5 Å are not included.

Compound	R3.28	K7.35	R7.36	W25	K95	H5	R267	W102	Y6	Total # of Ionic/Polar Interactions
KI16425	2.50	<i>4.26</i>	4.38	<i>4.02</i>	2.46	<i>3.78</i>		<i>4.12</i>	<i>3.70</i>	5
DGTP	4.57	<i>3.15</i>	3.57	<i>3.67</i>	3.07	<i>3.01</i>	<u>4.26</u>	<i>3.07</i>	<u>4.23</u>	4
DGP	2.28	<u>4.25</u>	2.08	<u>2.84</u>	2.54	<u>3.43</u>		<i>4.27</i>		4

Ionic interactions identified by bold type

Complementary polar interactions identified by italic type

Hydrophobic interactions underlined

Table 2

Pharmacophore hits obtained using different anionic isosteres in pharmacophore searches of the NCI database.

Anionic group	Number of hits	Number docked in LPA ₃
Phosphate	58	21
Carboxylate	748	121
Sulfonamide	131	15
Sulfate	15	5
Sulfite	146	50
Totals	1098	212

Table 3

Distances between pharmacophore features derived using different LPA receptor complexes.

	LPA₃ antagonist Distances(Å)	LPA₁ antagonist Distances(Å)	LPA₃ agonist Distances(Å)
Anionic to hydrophobic	8–14 & 12–16	8–12 & 7–11	8–10 & 13–15
Hydrophobic to hydrophobic	7–12	6–10	4–5

Table 4
Inhibition of LPA-induced calcium responses by selected NCI pharmacophore hits.

Compound	LPA ₁	LPA ₂	LPA ₃	LPA ₄	LPA ₅
NSC343949	No effect	No effect	No effect	NT ^c	NT ^c
NSC161613	No effect	No effect	IC ₅₀ 24 nM I _{max} =69.8% ^a	No effect	No effect
NSC255523 (NBAP)	No effect	No effect	Potentiator	NT ^c	NT ^c
NSC48776	No effect	No effect	No effect	NT ^c	NT ^c
NSC1741	IC ₅₀ >7.3μM I _{max} =59.3% ^a	IC ₅₀ >9.4μM I _{max} =88.0% ^b	I _{max} =53.7% ^a	NT ^c	NT ^c
NSC18749	EC ₅₀ >12.3μM E _{max} =180 at 30μM	EC ₅₀ >11.4μM E _{max} : 113 at 30μM	EC ₅₀ >11.8μM E _{max} 214 at 30μM	NT ^c	NT ^c
NSC47091	No effect	IC ₅₀ 355 nM I _{max} =53.3% ^b	IC ₅₀ 30 nM I _{max} =81.7% ^a	NT ^c	NT ^c

^a Inhibition of 200nM LPA response

^b Inhibition of 10nM LPA response

^c NT: Not tested

Table 5

Interaction distances between pharmacophore hits experimentally screened and LPA₃ receptor residues. Interactions with distances > 4.5 Å are not included.

Compound (LPA ₃ activity)	R3.28	K7.35	R7.36	W25	K95	H5	R267	W102	Y6	Sum of Ionic/Polar Interactions
NSC161613 (IC ₅₀ =24 nM)	1.37		1.27	<u>3.72</u>	1.20	1.72	1.62	<u>2.56</u>	3.57	6
NSC47091 (IC ₅₀ =30 nM)		1.27		<u>3.63</u>	1.40	4.27	1.38	<u>2.64</u>		4
NSC1741 (I _{max} =53.7% @ 200 nM)	2.39	3.91	3.61		3.8	3.78	3.61	4.41	4.37	8
NSC48776 (No Effect)	2.47		2.80	<u>3.66</u>	2.39	2.79	3.33		<u>3.56</u>	3
NSC343949 (No Effect)	3.70	<u>2.81</u>		<u>3.87</u>		<u>2.50</u>		<u>3.54</u>		1

Table 6
Pharmacological profiles of hit2lead compounds screened based on similarity to NSC47091.

Compound	LPA ₁	LPA ₂	LPA ₃	LPA ₄	LPA ₅
5105099	IC ₅₀ = 220 nM ^a K _i = 50 nM	IC ₅₀ = 21.8 nM K _i = 20.8 nM	No effect	NT	NT
6637041	I _{max} = 34.6% ^d at 30μM	I _{max} = 46.0% ^b at 30μM	IC ₅₀ = 347 nM K _i = 316.6 nM	NT	NT
5226501	I _{max} = 59.0% ^d at 30μM	IC ₅₀ = 28.3 nM K _i = 21.1 nM	IC ₅₀ = 13850 nM K _i = 7020 nM	NT	NT
5186303	IC ₅₀ = 27354 nM K _i = 13470 nM	IC ₅₀ = 8.9 nM K _i = 7.2 nM	IC ₅₀ = 1230 nM K _i = 310 nM	NT	NT
6636019	I _{max} = 52.2% ^a at 30μM	No effect	I _{max} = 16.0% ^a at 30μM	NT	NT

^a Inhibition of 200nM LPA response

^b Inhibition of 25nM LPA response

^c NT: Not tested