Published in final edited form as:

ChemMedChem. 2011 May 2; 6(5): 922–935. doi:10.1002/cmdc.201000425.

Benzyl and Naphthalene-Methyl Phosphonic Acid Inhibitors of Autotaxin with Anti-invasive and Anti-metastatic Actions

Dr. Renuka Gupte^[a], Dr. Renukadevi Patil^[a], Dr. Jianxiong Liu^[b], Dr. Yaohong Wang^[b], Dr. Sue C. Lee^[b], Dr. Yuko Fujiwara^[b], James Fells^[b], Dr. Alyssa L. Bolen^[b], Dr. Karin Emmons-Thompson^[a], Prof. C. Ryan Yates^[a], Dr. Anjaih Siddam^[a], Dr. Nattapon Panupinthu^[c], Dr. Truc-Chi T. Pham^[d], Prof. Daniel L. Baker^[d], Prof. Abby L. Parrill^{[d],[e]}, Prof. Gordon B. Mills^[c], Prof. Gabor Tigyi^[b], and Prof. Duane D. Miller^[a]

Duane D. Miller: dmiller@uthsc.edu

^[a]Department of Pharmaceutical Sciences, College of Pharmacy, University of Tennessee Health Science Center, Memphis, TN 38163 (USA) Fax: (+1) 901 448 3446

^[b]Department of Physiology, College of Medicine, University of Tennessee Health Science Center, Memphis, TN 38163 (USA)

^[c]Department of Systems Biology, The University of Texas MD Anderson Cancer Center, Houston, TX 77054 (USA)

^[d]Department of Chemistry, University of Memphis, TN 38152 (USA)

^[e]Computational Research on Materials Institute, University of Memphis, TN 38152 (USA)

Abstract

Autotaxin (ATX, NPP2) is a member of the nucleotide pyrophosphate phosphodiesterase enzyme family. ATX catalyzes the hydrolytic cleavage of lysophosphatidylcholine (LPC) via a lysophospholipase D activity that leads to the generation of the growth factor-like lipid mediator lysophosphatidic acid (LPA). ATX is highly upregulated in metastatic and chemotherapy-resistant carcinomas and represents a potential target to mediate cancer invasion and metastasis. Here we report the synthesis and pharmacological characterization of inhibitors of ATX based on the 4-tetradecanoylamino benzyl phosphonic acid scaffold that was previously found to lack sufficient stability in cellular systems. The new 4-substituted benzyl phosphonic acid and 6-substituted naphthalen-2-yl-methyl phosphonic acid analogs blocked ATX with K_i values in the low-micromolar-nanomolar range against FS-3, LPC, and nucleotide substrates through a mixed-mode mechanism of inhibition. None of the compounds tested inhibited the activity of related enzymes (NPP6 and NPP7). In addition, the compounds were evaluated as agonists or antagonists of seven LPA receptor subtypes. Analogs **22** and **30b**, the two most potent ATX inhibitors, dose-dependently inhibited the invasion of MM1 hepatoma cells across murine mesothelial and human vascular endothelial monolayers *in vitro*. The average terminal half-life for compound **22** was 10h $\pm$ 5.4h and it caused a long-lasting reduction plasma LPA levels. Compounds **22** and **30b** significantly reduced lung metastasis of B16-F10 syngeneic mouse melanoma in a post-inoculation treatment paradigm. The described 4-substituted benzyl phosphonic acids and 6-substituted naphthalen-2-yl-methyl phosphonic acids represent new lead compounds that effectively inhibit the ATX-LPA-LPA receptor axis both *in vitro* and *in vivo*.

Keywords

ATX inhibitors; LPA receptors; 4-substituted benzyl phosphonic acids; 6-substituted naphthalen-2-yl-methyl phosphonic acids; structure-activity relationships

Introduction

Autotaxin (ATX, NPP2) is member of the nucleotide pyrophosphate phosphodiesterase (NPP) enzyme family. ATX catalyzes the hydrolysis of lysophosphatidylcholine (LPC) via a lysophospholipase D (LPLD) activity that leads to the generation of the growth factor-like, lipid mediator lysophosphatidic acid (LPA).^[1, 2] ATX is a 125 kDa glycoprotein, originally isolated from human melanoma cells, that stimulates tumor cell motility and has been implicated in the metastatic and invasive properties, as well as chemotherapeutic resistance of many carcinomas.^[3–12] NPP6 and NPP7 are the only other known NPP isoforms that utilize lysophospholipids as substrates. However, In contrast to ATX, NPP6 and NPP7 act as lysophospholipase C. NPP6 cleaves phosphocholine from LPC, sphingosylphosphorylcholine and glycerophosphorylcholine to generate monoacylglycerol, sphingosine and glycerol, respectively.^[13] NPP7, also referred to as alkaline sphingomyelinase hydrolyzes sphingomyelin to generate ceramide in the intestinal tract but also cleaves phosphocholine from LPC and platelet activating factor to generate monoacyl and alkyl-acetyl glycerols.^[14, 15]

ATX is required for normal development. Homozygous ATX knockout mice die in utero at day 9.5 coinciding with a period of vascular stabilization.^[16, 17] Likewise, ATX plays an important role in the development of the nervous system as ATX knockout mice show defects in neural tube development.^[18] LPA is a mitogen, motogen, and anti-apoptotic agent, which provides survival advantages to carcinomas that utilize LPA in an autocrine or paracrine fashion. Ovarian cancer cells produce high levels of LPA.^[19–21] Copy numbers increase in ovarian cancers in chromosomal region 8q24, which contains the genes encoding for ATX and the Myc oncogene.^[22] Euer *et al.* found that ATX was among the 40 most upregulated genes in highly metastatic cancers.^[23] Recently, it has been shown that ectopic expression of ATX in mice leads to mammary intraepithelial neoplasia, which develops into invasive and metastatic tumors.^[24] ATX inhibits paclitaxel-induced apoptosis in breast cancer cells,^[6] and LPA renders ovarian cancer cells chemoresistant to cisplatin and adriamycin.^[25] ATX is also overexpressed in patients with recurrent disease after prior treatment with chemotherapy.^[26] In a genome-wide siRNA screen, we identified ATX as a candidate drug-resistance gene in ovarian cancer.^[7] In addition we showed that a lipid-like, small-molecule inhibitor of ATX, carba-cyclic-phosphatidic acid increases the sensitivity of resistant ovarian cancer cells to paclitaxel treatment.^[7]

The ATX-LPA-LPA receptor axis is a promising therapeutic target for the management of cancer metastasis and therapeutic resistance. ATX shows feedback inhibition by its hydrolysis products LPA, CPA, and sphingosine-1-phosphate (S1P).^[27, 28] Many initially identified ATX inhibitors are lipid-like substrate or product analogs.^[29–36] The characteristics of this type of compounds limit their utility as potential lead compounds for drug development. Non-lipid ATX inhibitors have also been identified but most of these compounds lack sufficient stability and characterization in tumor models.^[31, 37–42] Recently, Ferry and colleagues described an ATX inhibitor 4-tetradecanoylamino benzyl phosphonic acid (S32826, Figure 1) that possessed nanomolar activity *in-vitro*.^[43] Unfortunately, S32826 failed to show activity in cellular and *in-vivo* systems. We hypothesized that hydrolysis of the amide bond present in S32826 could be the reason for its instability and thus lack of activity in cellular systems.

To overcome the presumed lack of stability of S32826, we designed analogs that were expected to be more stable than the parent compound. We have generated a panel of analogs that inhibit ATX with potencies comparable to that of S32826. These stabilized analogs inhibit ATX via a mixed-mode mechanism *in vitro* without any effect on the related lysophospholipid phosphodiesterases, NPP6 and NPP7 or on LPA receptors (LPAR). Two of these compounds, **22** and **30b** inhibited ATX-dependent invasion of rat MM1 hepatoma cells of mesothelial cell and human umbilical cord vascular endothelial cell (HUVEC) monolayers *in vitro*. In addition, **22** and **30b** showed a profound reduction in lung foci *in vivo* using the B16-F10 syngeneic melanoma metastasis model in C57BL/6 mice. Based on these results compounds **22** and **30b** represent promising leads for further synthetic improvement and also provide proof of principle that ATX inhibitors offer therapeutic utility in the control of cancer metastasis *in vivo*.

Results and Discussion

Synthesis

The synthesis 4-(hexadecane-1-sulfonylamino)benzyl phosphonic acid (**4**) is depicted in Scheme 1. Commercially available 1-hexadecanesulfonyl chloride (**2**) was added to a mixture of 4-aminobenzylphosphonic acid diethyl ester (**1**) and *N,N*-diisopropylethylamine in THF under reflux conditions to give compound **3**, which was deprotected with bromotrimethylsilane (TMSBr) followed by addition of MeOH under stirring for 30 min to afford compound **4**. The synthesis of compound **10** started with alkylation of 4-hydroxymethylphenol (**5**) (Scheme 2) using 1-bromotetradecane (**6**) in the presence of K₂CO₃ and 18-crown-6 to get compound **7** followed by treatment with PBr₃ to obtain **8** in 98% yield. The Michealis–Arubuzov reaction^[44] on compound **8** in trimethyl phosphate provided compound **9**, which was then deprotected using TMSBr in anhydrous acetonitrile to yield **10**.

Scheme 3 demonstrates the synthesis of (*E*)-4-(pentadec-1-enyl)benzylphosphonic acid (**18**) and 4-pentadecylbenzylphosphonic acid (**22**). (*E*)-Methyl 4-(pentadec-1-enyl)benzoate (**13**) was synthesized by the Heck coupling of **11** and **12** using Palladium (II) acetate, and triethyl amine in anhydrous DMF. Compound **13** was reduced using LAH in THF at 0 °C to room temperature to produce **14** and also saturated under catalytic hydrogenation conditions to generate compound **15**. Bromination of Compound **14** with PBr₃ in ether gave **16**, which was then subjected to The Michealis–Arubuzov reaction^[44] using trimethyl phosphite to get **17**. Compound **17** was treated with TMSBr in acetonitrile followed by addition of MeOH to furnish **18**. Compound **22** was synthesized utilizing the same approach starting from compound **15**.

The synthesis of 6-substituted naphthalen-2-yl-methyl phosphonic acid analogs (**30a–b** & **34a–b**) was performed using the same procedure described for the synthesis of compounds **18** and **22** (Scheme 4). We then focused our medicinal chemistry efforts on phosphomimetic functional groups, the phosphomimetics include compounds **37**, **41**, **42**, and **43** (Scheme 5). We started with the conversion of compound **19** into an aldehyde derivative **35** using pyridinium dichromate (PDC) in CH₂Cl₂. The resulting aldehyde **35** was then converted to the α-hydroxy phosphonate derivative **36** using the Pudovik reaction.^[34, 45] Compound **36** was deprotected using TMSBr to produce target compound **37**. Compound **36** was treated with DAST in ether, SOCl₂/CH₂Cl₂ and PBr₃/ether to obtain the desired compounds **38**, **39**, and **40**, respectively. Finally, the compounds **38**, **39**, and **40** were treated with TMSBr followed by addition of MeOH to afford compounds **41**, **42**, and **43** in good yields.

The synthesis of target compounds **45** and **47** is outlined in Scheme 6. Compound **19** was treated with a mixture of 1*H*-tetrazole and bis(2-cyanoethyl)-*N,N*-diisopropylphosphoramidite in anhydrous CH₂Cl₂ followed by the addition of hydrogen peroxide to give the bis-cyanoethyl protected fatty alcohol phosphate **44**. Removal of the cyano ethyl groups with treatment of methanolic KOH followed by acidification furnished the phosphate **45**. Similarly **19** was treated with a mixture of 1*H*-tetrazole and bis(2-cyanoethyl)-*N,N*-diisopropylphosphoramidite in anhydrous CH₂Cl₂ followed by reflux in the presence of elemental sulfur to give bis-cyanoethylprotected fatty alcohol thiophosphate **46**, which, in turn, was treated with methanolic KOH followed by acidification to yield the thiophosphate **47**.

Biological Results

Effect of 4-substituted benzyl phosphonic acid and 6-substituted naphthalene-2-yl-methyl phosphonic acid derivatives on ATX and LPAR—The first level of testing was done using 10 μM concentrations of the compounds added to 2 nM recombinant human ATX and the FRET substrate FS-3. After a 2h incubation the amount of FS3 hydrolyzed was measured and the residual ATX activity was expressed as a percent of the vehicle-treated sample less the autolysis of FS3 in the absence of ATX. In this assay, S32826 reduced the amount of FS3 hydrolyzed by 97% (Table Ia& b). Among the 4-substituted benzyl phosphonic acid analogs compound **22** showed 95% inhibition of FS3 hydrolysis (Table Ia), whereas compound **30b** from the 6-substituted naphthalene-2-yl-methyl phosphonic acid series reduced FS3 hydrolysis by 83% (Table Ib). Dose-response curves were generated with compounds S32836, **22** and **30b** and compared to the feedback inhibition of the ATX product LPA (Figure 2). Each of these three compounds dose-dependently and completely inhibited ATX.

Subsequently, we generated analogs of compounds **22** and **30b** and examined their inhibition of ATX. The modifications were either in the aliphatic chain, the linker of the phosphate moiety, or on the phosphate moiety itself (Table Ia & b).

The most effective analogs based on the single 10-μM dose inhibition assay were extensively characterized to determine IC₅₀, K_i, and K_i' values against ATX-mediated hydrolysis of FS3. Replacement of the amide with a sulfonamide (**4**) reduced potency. Introduction of an ether (**10**) or alkene (**18**) connecting the hydrocarbon chain to the benzyl ring decreased the potency compared to the simple aliphatic chain. Linkage of the phosphate moiety in compound **45** through a methyl phosphonate reduced efficacy. However, converting the phosphate to a thiophosphate (**47**) in this scaffold increased efficacy at 10 μM 5.6-fold. α-Halogenation of the linker methyl group generally decreased potency of compounds **41**, **42**, and **43** relative to compound **22**. Insertion of a α-hydroxyl group into the linker methylene increased the potency of this analog but it did not surpass that of compound **22**.

Modification of the side chain at position 6 of the naphthalene-2-yl-methyl phosphonic acid scaffold showed that the 10-carbon sidechain was more effective than the 11 carbon chain and that incorporation of an alkene had differential effects depending on the chain length (decreased efficacy of compound **30a** relative to **34a**, but increased efficacy of **30b** over **34b**). Analysis of the seven analogs showed that compound **22** in the benzyl phosphonic acid series and compound **30b** in the naphthalene-methyl phosphonic acids were the most potent analogs with respect to ATX inhibition.

Because FS-3 is not a natural substrate of ATX it was determined whether the inhibitors also blocked LPA production from a fluorescent analog of LPC(3-acyl-7-dimethylaminonaphthyl-1-LPC, ADMAN-LPC) and the hydrolysis of the nucleotide-like

substrate p-nitrophenyl thymidine 5'-monophosphate (pNP-TMP). Compound **22** and **30b** inhibited LPA production from ADMAN-LPC and the hydrolysis of the pNP-TMP substrate (Figure 3). In order to determine the mechanism of ATX inhibition, K_i (affinity of the compound for free enzyme) and K_i' (affinity of the compound for the enzyme substrate complex) were determined using simultaneous nonlinear fits of the Michaelis-Menten equations for competitive, uncompetitive, mixed-mode, and non-competitive inhibition as we have previously described.^[37, 40, 46] These experiments uniformly showed that these compounds displayed similar K_i and K_i' values which is consistent with a mixed-mode type mechanism of inhibition.

ATX inhibitors have been examined against other NPP family members.^[30, 37, 38] NPP6 and NPP7 are the only NPP isoforms beyond ATX that are known to utilize lysophospholipid phosphodiester as substrates. Thus, specificity was addressed by determining the activity of NPP6 and NPP7 in the presence and absence of these analogs. None of the analogs tested blocked the activity of NPP6 or NPP7 by greater than 10% at a single 10 μ M dose (Tables I a and I b).

Some inhibitors of ATX, including LPA phosphonates^[29] and LPA bromophosphonates,^[35] have been shown to inhibit ATX and block LPA receptor subtypes. LPA bromophosphonate (LPA-BP) was also shown to inhibit cancer metastasis and reduced tumor size in mice.^[35] For this reason, we examined whether the two leads **22** and **30b** affected the activation of multiple LPA GPCR targets using cell lines overexpressing individual LPA GPCR coupled to Ca^{2+} mobilization as we have previously described.^[35] Analyses were done using 10 μ M concentrations of the test compounds alone or in combination with an EC_{50} concentration of LPA for the given receptor subtype to determine agonist and antagonist activity, respectively (Table II).

Some of the compounds that showed > 10% attenuation of the LPA response or activation > 10% of LPA at a given receptor subtype at 10 μ M were subjected to dose-response/inhibition experiments. Unexpectedly, compounds **45** and **47** were weak but full agonists at LPA_5 with EC_{50} values of 7.9 μ M and 2.9 μ M, respectively. Compound **47** was also a partial agonist at LPA_3 ($E_{max_{10\mu M}} \sim 44\%$ of the maximal LPA response). Compound **45** caused a 50% reduction in the LPA_2 response when applied at 10 μ M. Compound **33b** elicited a 33% activation of LPA_3 and a 17% activation at LPA_5 when applied at 10 μ M. Likewise, compound **22** (10 μ M) was a weak partial agonist at LPA_2 ($E_{max_{10\mu M}} \sim 20\%$), whereas analogs **37** and **41** inhibited this receptor subtype by 28% and 26%, respectively. Based on these findings we conclude that these analogs were poor ligands of the LPA GPCR tested with the exception of compounds **45** and **47**, which were full agonists of LPA_5 albeit with low potency. At the present time, very few LPA_5 selective compounds have been described in the literature^[47] hence the identification of these two hits might serve as a starting point for a more comprehensive characterization of the structure-activity relationship of this receptor subtype.

ATX inhibitors block hepatocarcinoma invasion of mesothelial and HUVEC monolayers—Cancer metastasis is a complex process that cannot be accurately modeled *in vitro*. Models that utilize cellular monolayers for the invasion of carcinoma cell come the closest to the situation *in vivo*.^[48–51] Here we used two different cell monolayers, mouse mesothelium and human vascular endothelium to examine the role of ATX in the invasion of MM1 hepatocarcinoma cells. The mesothelial monolayer is an accepted model for invasion of body cavities lined by serous cells whereas, the HUVEC monolayer is considered to be an *in vitro* model of hematogenous invasion of carcinomas.

First we determined if exposure to compounds **22**, **30b** and the combined ATX inhibitor LPA receptor pan-antagonist compound LPA-bromophosphonate^[35] caused toxicity *in vitro*. We exposed the cells of the mesothelium monolayer or the MM1 cell line to these 10 μ M compounds dissolved in 1% DMSO for 20h and examined cell viability using the trypan blue dye exclusion assay. Neither compound caused a decrease in cell viability compared to the vehicle and all viabilities were greater than 93%.

The mesothelial and HUVEC cells of the monolayer and the invading MM1 cells express different levels of ATX (Figure 4 A–C). Quantitative real-time PCR performed with mRNA isolated from MM1, mesothelium and HUVEC cells showed that the primary source of ATX is the MM1 cell as the other two cell types showed very low expression. MM1 cells showed robust expression of transcripts encoding the P2Y family of LPA receptors and LPA₂ in the EDG receptor subfamily. In mesothelial cells LPA₂ is the predominant receptor whereas, in HUVECs it is the LPA₅ receptor.

Addition of LPC to the co-culture increases invasion in a dose-dependent manner (Figure 4D). We hypothesized that inhibition of ATX by our two lead compounds should attenuate LPA production *in situ* and reduce invasion of MM1 carcinoma cells through the mesothelial and HUVEC monolayers. Compounds **22** and **30b** were applied with or without LPC to the co-cultures and the number of invading cells was quantified after 24 h of the co-culture (Figure 4E). Both compounds inhibited MM1 cell invasion reaching a complete inhibition of LPA and likely ATX-dependent invasion above 3 μ M. The two compounds were also tested for their ability to inhibit MM1 cell invasion of HUVEC monolayers (Figure 4F). Similarly to that seen for the invasion of murine mesothelial monolayers, compounds **22** and **30b** dose-dependently inhibited the LPC-dependent invasion of the HUVEC monolayer, whereas the compounds alone did not reduce the basal rate of invasion. We have conducted similar experiments with rat microvascular cell monolayers and found similar inhibition und **22** and **30b** although the number of invading cells per field was considerably lower than that in HUVEC or mesothelial monolayers (data not shown). These results provide evidence that the inhibition of ATX *in situ* can fully inhibit LPC-dependent invasion of carcinoma cells suggesting the potential applicability of our compounds in animal models of carcinoma metastasis.

Pharmacokinetic characterization of compound 22—Because of the slightly higher potency of compound **22** inhibiting ATX we selected this analogue for preliminary pharmacokinetic characterization. The plasma concentration versus time profile of compound **22** declined biexponentially, exhibiting initial distribution and terminal elimination phases (Figure 5). Non-compartmental analysis following intravenous administration showed that volume of distribution (Vd) for compound **22** was 3.9 L/kg, and the clearance was estimated to be 8.6 ± 0.5 mL/min/kg. The average terminal half-life for compound **22** was $10\text{h} \pm 5.4\text{h}$ with a mean plasma residence time of 7.6 ± 0.5 h.

LPA levels were monitored simultaneously for the 16:0, 18:1, 18:2 and 20:4 acyl species and showed similar changes regardless of the fatty acyl chain length. Plasma LPA levels showed a rapid decrease with a nadir at 60 min followed by a transient rise at 120 min and second decrease reaching a minimum in the 480 min sample. LPA levels remained depressed at approximately 50% of the pretreatment level in the 1440 min sample, the longest sampling time included in the study. These results indicate a long-lasting decrease in plasma LPA that appears to be consistent with the 10h $T_{1/2}$ of this compound. Of note is a confounding factor that at 2h 50 IU heparin was injected to keep the cannulas open that may have affected plasma LPA levels. Furthermore, these results may not be reflective of the levels following intraperitoneal administration used in the murine metastasis model. Further experiments will be needed to characterize the pharmacokinetic properties of compound **22**

and other analogues using different routes of administration. It is important to recognize that plasma LPA levels are not likely to be indicative of the LPA level in the tumor microenvironment as supported by the lack of elevation in LPA or ATX activity in different cancers with the exception of follicular lymphoma.^[8, 19, 42, 52, 53]

ATX inhibitors reduce B16 melanoma metastasis in vivo—We conducted an acute toxicity study with compound **22** by exposing Swiss mice to 1 – 10 – 30 mg/kg/day doses for 10 days. We found no noticeable change in animal behavior within 1 h after injection or weight loss. No death was detected in any of the groups. Necropsy conducted on day 14 showed no noticeable organ damage with the exception of adhesions and splenomegaly in three mice in the group receiving the highest dose. We conclude that the non-observable adverse effect level of compound **22** is > 10 mg/kg.

Inhibitors of ATX have been shown to reduce tumor metastasis in different animal models.^[32, 35] Here we applied the syngeneic B16-F10 mouse melanoma model of hematogenous lung metastasis in C57BL/6 mice that we previously showed responds to inhibitors of ATX.^[32] C57BL/6 mice were inoculated with B16-F10 melanoma cells via the tail vein. Beginning 30 min later the mice were treated with either 0.5 mg/kg/day of the two lead compounds or vehicle (PBS with 1% DMSO) via the intraperitoneal route for an additional 10 days. As a positive control, we also applied LPA-BP that we showed previously to inhibit the metastasis of breast and colon cancers in xenograft models.^[35, 54] On day 21 the animals were sacrificed and the lungs were isolated and metastatic nodules in the lungs were quantified. In this metastasis model, compounds **22** and **30b** significantly reduced the number of metastatic foci and their efficacy at this dose was comparable to that of LPA-BP (Figure 6A & B).

In the present study we provide evidence for the potential therapeutic utility of ATX inhibitors derived from the benzyl phosphonic acid scaffold. We have modified and expanded this scaffold to 6-substituted naphthalene-methyl phosphonic acid derivatives that were also effective inhibitors of ATX. Our limited structure-activity analysis showed that some of these compounds are high potency inhibitors of ATX without significant effects on LPA GPCR in the nanomolar and low micromolar range. Unlike the parent compound S32826 that lacked activity in cellular assays,^[43] these novel analogs showed strong inhibition of ATX-mediated tumor cell invasion of mesothelial and endothelial cell monolayers and were as effective *in vivo* as the previously reported dual action ATX inhibitor and LPA GPCR antagonist LPA-BP.^[32, 35, 54]

There is increasing interest in developing inhibitors of ATX to control the tumor promoting and pro-inflammatory roles of LPA.^[30, 31, 35, 37–40, 55] Carba cyclic-phosphatidic acid was the first ATX inhibitor scaffold explored to control carcinoma invasion and metastasis *in vivo*.^[32] Carba cyclic-phosphatidic acid inhibited ATX without activating LPA_{1–4}. However, these compounds activate LPA₅ that complicates their utility.^[47] Subsequently, the sn-1 or sn-2 hydroxy groups of LPA have been replaced by fluorine, difluoromethyl, difluoroethyl, O-methyl or O-hydroxyethoxy groups to give non-migrating LPA analogues that resist acyltransferases^[56] and some of these compounds, including LPA-BP, have been found to possess dual action by inhibiting ATX and LPA GPCR.^[35] LPA-BP inhibited breast cancer metastasis and tumor growth *in vivo*.^[35] Several new ATX inhibitor scaffolds have been reported recently;^[30, 31, 37–40, 55] however, these compounds have not been evaluated against other members of the NPP family and were not explored for their ability to control invasion and/or metastasis. Our study for the first time provides proof of the principle that lipid-like ATX inhibitors are capable of controlling cancer metastasis *in vivo* and also validate the monolayer invasion assay as a valuable cell-based screening for antimetastatic compounds.

The preclinical screening template consisting of assaying the activity of analogs against ATX, followed by some related NPP isoforms, LPA GPCR, monolayer invasion assays, followed by the syngeneic animal metastasis model offers a comprehensive stepwise protocol for the identification of ATX inhibitors with utility to control tumor progression. Given the relatively short half-life of ATX^[57] and the high rate of LPA turnover *in vivo*^[31] we foresee the need of compounds with long plasma half-life and broad tissue distribution to control the constitutive production of ATX and the generation of LPA. The present data derived from cellular and *in vivo* experiments suggest that further modification of the benzyl and naphthyl phosphonic acid scaffolds might yield even more suitable derivatives for the control of carcinoma metastasis. Pharmacokinetic profiling of other analogues will be a crucially important step in further evaluation and preclinical development of ATX inhibitors for the identification of scaffolds that exert long-term inhibition of ATX in tumor microenvironment.

Conclusion

Two autotaxin inhibitors **22** and **30b** were identified to be potent and stable in in-vivo models. All of the compounds synthesized were specific for NPP-2/ ATX. The mode of enzyme inhibition of the most potent compounds was determined and was found to be mixed. The fact that the ATX inhibitors blocked hepatocarcinoma invasion of mesothelial and HUVEC monolayers bestows evidence that the inhibition of ATX *in situ* can fully inhibit LPC-dependent invasion of carcinoma cells signifying the potential applicability of our compounds in animal models of carcinoma metastasis. Our compounds **22** and **30b** have shown outstanding *in vivo* profile by reducing the lung metastases of B16-F10 syngeneic mouse melanoma in a post-inoculation treatment model.

Experimental section

Chemistry

All starting materials, reagents and solvents were obtained from commercial suppliers and were used without further purification. Reactions were performed under an inert atmosphere of nitrogen, unless otherwise specified. Routine thin-layer chromatography (TLC) was performed on silica gel plates (Analtech, Inc., 250 microns). Flash chromatography was conducted on silica gel (Merck, grade 60, 230–400 mesh). ¹H NMR spectra were recorded on a Bruker ARX 300 spectrometer (300 MHz) or Varian spectrometer (500MHz) using DMSO-*d*₆ and CDCl₃ as solvents, and spectral data were consistent with assigned structures. Chemical shift values were reported as parts per million (δ), coupling constants (*J*) are given in Hz, and splitting patterns are designated as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet. Mass spectra were collected on a Bruker ESQUIRE electrospray/ion trap instrument in the positive and negative modes. High-resolution mass measurements were obtained using a Micromass Q-TOF2 mass spectrometer. Elemental analyses (C, H, N) was performed by Atlantic Microlab, Inc. (Norcross, GA), and results were within $\pm 0.4\%$ of the theoretical values for the formula given.

General procedure for synthesis of Phosphonic acid analogs (Compounds **4**, **10**, **18**, **22**, **30a**, **30b**, **34a**, **34b**, **37**, **41**, **42** and **43**) (GP 1)

To a suspension of phosphonate derivative (1 equiv) in anhydrous acetonitrile, TMSBr (2.5 equiv) was added and the reaction mixture was refluxed for 1 h. The acetonitrile was then evaporated under reduced pressure and MeOH was added to the residue and stirred for 30 min at RT. The desired product was obtained in good yield after filtration of MeOH solution as a white solid.

4-Pentadecylbenzylphosphonic acid (22)—Yield (0.153 g, 53%); ^1H NMR (500 MHz, $[\text{D}_6]$ DMSO): δ = 7.139–7.063 overlapping protons (m, 4H); 2.91(d, 2H, J =20 Hz); 1.52 (m, 2H); 1.26(m, 2H); 1.15(s, 24H); 0.853 (t, 3H); ^{13}C NMR (500 MHz, $[\text{D}_6]$ DMSO): δ = 140.2, 131.6, 129.8, 128.4, 38.1, 35.1, 31.8, 29.5, 29.3, 29.2, 22.6, 14.2 ppm; MS (ESI) m/z 381.0 $[\text{M}-\text{H}]^-$; Anal Calcd. for $\text{C}_{22}\text{H}_{39}\text{O}_3\text{P}$; C, 69.08; H, 10.28; Found C, 69.33; H, 10.52.

General procedure for synthesis of compounds 8, 16, 20, 28a, 28b, 32a, 32b and 40 (GP 2)

PBr_3 (0.3 equiv) was added slowly under stirring to a solution of alcohol derivative (1 equiv) in anhydrous CH_2Cl_2 at 0°C . The reaction mixture was stirred at RT for 1 h and the formation of the product was monitored by TLC. Water was added at 0°C to quench the reaction, extracted from CH_2Cl_2 and dried over MgSO_4 . The product mass confirmed by mass spectroscopy and the residue was carried on to the next step without further purification.

1-Bromomethyl-4-pentadecyl-benzene (20)—MS (ESI) m/z 380 $[\text{M}-\text{H}]^-$.

General procedure for synthesis of compounds 9, 17, 21, 29a, 29b, 33a and 33b (GP 3)

Trimethylphosphite (9 equiv) was added to dry bromide derivative (1 equiv) at RT and the mixture was refluxed for 18 h. The trimethylphosphite was evaporated under high vacuum overnight and the crude residue was purified by column chromatography ($\text{CHCl}_3/\text{MeOH}$ 3:1) to give pure compound in quantitative yield.

Dimethyl-4-pentadecylbenzylphosphonate (21)— ^1H NMR (300 MHz, CDCl_3) δ = 7.4–7.0 overlapping protons (m, 4H); 3.66–3.63 (d, 6H, J = 15 Hz); 3.2 (d, 2H); 2.6 (m, 2H); 1.6 (m, 2H); 1.2 (s, 24H); 0.895 (t, 3H). MS (ESI) m/z 433 $[\text{M}+\text{Na}]^+$.

General procedure for synthesis of compounds 13, 25a and 25b (GP 4)

To a mixture of aryl bromide derivative (1 equiv), Palladium(II) acetate (5 mol %), and triethyl amine (1 equiv) in anhydrous DMF was added substituted alkene (1 equiv) successively. The reaction mixture was refluxed for 16 h, filtered on bed of celite and extracted with ethylacetate and water. The compound was purified by flash column chromatography using ethyl acetate and hexane (1:2) mixture.

(E)-Methyl-6-(dodec-1-enyl)-2-naphthoate (25a)— ^1H NMR (300 MHz, $[\text{D}_6]$ DMSO): δ = 8.582–7.771 overlapping protons (6H); 6.65–6.52 (m, 1H); 5.7–5.2 (m, 1H); 3.91–3.90 (s, 3H); 2.1–1.8 (m, 2H); 1.76–1.55(m, 2H); 1.209(s, 14H); 0.856 (t, 3H). MS (ESI) m/z 375.3 $[\text{M}+\text{Na}]^+$.

General procedure for synthesis of Compound 14, 19, 26a, 26b, 31a and 31b (GP 5)

Anhydrous THF was added to LAH (3 equiv) and stirred for 5 min. To this, a solution of corresponding methyl ester (1 equiv) in THF was added and the mixture was stirred at RT for 4 h. The reaction mixture was cooled to 0°C and saturated sodium sulfate was added drop wise to the mixture. The product was then extracted with ethyl acetate and the crude product was purified using flash column chromatography which was eluted with ethyl acetate and hexane mixture (1:1).

(E)-6-(Dodec-1-enyl)naphthalen-2-yl-methanol (26a)— ^1H NMR (300 MHz, CDCl_3) δ = 7.9–7.3 overlapping protons (6H); 6.6–6.3 (m, 1H); 5.6–5.4 (m, 1H); 4.814(s, 2H); 2.35–2.26 (m, 2H); 1.6–1.47(m, 2H); 1.268(s, 14H); 0.9 (t, 3H). MS (ESI) m/z 347.3 $[\text{M}+\text{Na}]^+$.

General procedure for the synthesis of compounds 15, 27a and 27b (GP 6)

To a solution of the corresponding alkene of the methyl ester derivative (1 equiv) in MeOH was added catalytic amount of Pd(OH)₂/C and the mixture was stirred at RT for 2 h using catalytic amount of hydrogen gas. The completion of reaction was monitored by TLC and the reaction mixture was filtered on a bed of celite. The filtrate was evaporated, product mass was confirmed by mass spectroscopy and the crude residue was directly used for next step.

6-Dodecyl-naphthalene-2-carboxylic acid methyl ester (27a)—MS (ESI) m/z 355 [M+H]⁺.

Biology

Lysophosphatidic acid (18:1), LPC 18:1, and S1P were purchased from Avanti Polar Lipids (Alabaster, AL). For calcium mobilization assays, LPA, S1P, and the test compounds were prepared as 1 mM stock solutions in phosphate-buffered saline (PBS) in an equimolar complex with charcoal-stripped, fatty acid free bovine serum albumin (BSA; Sigma, St. Louis, MO). The fluorescent ATX substrate FS-3 was purchased from Echelon Biosciences (Salt Lake City, UT).

Autotaxin Inhibition Assays—As the first level of screen, 50 µl of recombinant-ATX (2 nM in final concentration) 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 (Echelon Biosciences Biosciences, Inc., Salt lake city, UT; final concentration 1 µM) and 25 µl of test compound dissolved in assay buffer with 40 µM bovine serum albumin (Sigma, St. Louis, MO) in 96-well Costar black-well plate. FS-3 fluorescence at excitation and emission wavelengths of 485 and 538 nm, respectively, were monitored using a FLEXstation II (Molecular Devices, Sunnyvale, CA) for 2 h of incubation at 37°C. The differences between time 0 and 120 min were calculated individually and normalized to the vehicle control. The mean ± SD of triplicate samples was expressed as percentage of ATX activity. The ATX activity in the presence of the test compounds was compared to vehicle using the Student's and p< 0.05 was considered significant.

The second level of screen was aimed at determining the inhibition of ATX activity against LPC substrate. We used the fluorescent analog ADMAN-LPC to measure the inhibition of LPA production as previously reported.^[32] ADMAN-LPC at a final concentration of 0.5 µM was mixed with or without the test compound at 3µM and 100nM ATX. The reaction incubated at 37 °C for 4 h and lipids were extracted and separated by Silica Gel 60 TLC plates (Merck Darmstadt) using chloroform/methanol/ammonium hydroxide (60:35:8 V/V/V). Fluorescent LPC and LPA species were visualized in a Photodyne UV imager using the TotalLab100 software.

The third level of screen utilized the nucleotide-like substrate pNP-TMP (Sigma-Aldrich, St. Louis, MO).^[32] The assay contained 4nM ATX, 5 mM pNP-TMP, and vehicle with or without the test compound (10 µM), all dissolved in 60 µL assay buffer. The assay was carried out in 96-well, half-area plates (Corning Inc., Corning, NY) at 37 °C in a BioTek Synergy-2 plate reader (BioTek, Winooski, VT) with absorbance monitoring at 405 nM.

Determination of the Mechanism of Autotaxin Inhibition—The mechanism of inhibition of ATX was determined using recombinant, purified human ATX and FS-3 as recently described.^[37, 38, 40] Final ATX and FS-3 concentrations were 8.3 nM and 1 µM, respectively and the assay buffer with 15 µM fatty acid free BSA. To calculate IC₅₀ full dose responses were determined for the test compounds. In addition, the mechanism of

inhibition of ATX-mediate hydrolysis of FS-3 was determined by varying the concentration (0.3 μ M to 20 μ M) of substrate in the presence of three concentrations of each inhibitor (0, 0.5 $\times$ and 2 $\times$ (IC_{50})). Kinetic data including V_{max} and K_m were determined using Kaleida Graph 4.0 (version 4.03, Synergy Software, Reading, PA) after the plots of initial velocities versus substrate concentration in the absence or presence of inhibitors were fit to the following equation $y = m_1 * m_2 * x / (1 + m_2 * x)$, where $K_m = 1/m_2$ and $V_{max} = m_1$. The average K_m for ATX-mediated FS-3 hydrolysis was determined to be 2.3 μ M and was used in the following calculations. Simultaneous non-linear regression using WinNonLin® 6.1 (Pharsight, Mountain View, CA) was used to assign the mechanism of inhibition.^[37, 38, 40] K_i and K_i' values (the affinity for free enzyme and enzyme substrate complex, respectively) were determined by calculating the lowest averaged percent residuals for each mechanism derived from curve fitting using the Michaelis-Menten equations for competitive, uncompetitive, mixed-mode, and non-competitive inhibition.

Calcium mobilization assays—Assays for mobilization of intracellular Ca^{2+} were performed as described.^[45, 58] Test compounds up to a final concentration of 10 μ M either alone or mixed with respective $\sim EC_{50}$ concentrations of LPA 18:1 for each of LPA receptor subtype were added to cells stably expressing LPA_{1,2,3,4,5}, GPR87, and P2Y10 LPA receptor subtypes.^[47] The cells were loaded with Fura-2/AM in Krebs buffer containing 0.001% pluronic acid for 1 h, and rinsed with Krebs buffer before measuring Ca^{2+} mobilization. The Ca^{2+} responses were measured using a Flex Station II fluorescent plate reader (Molecular Devices, Sunnyvale, CA). The ratio of peak emissions at 510 nm after 2 min of ligand addition was determined for excitation wavelengths of 340 nm/380 nm. All samples were run in triplicate, and assays were performed at least two times for each receptor.

NPP-6 and NPP-7 Inhibition Assay—Inhibition of NPP6 and NPP7 was evaluated using recombinant, purified proteins, the synthetic substrate *para*-nitrophenylphosphocholine (pNPPC) and a Synergy II plate reader (BioTek, Winooski, VT) as we have previously described.^[37, 40] The final concentration of each enzyme was 8.3 nM and pNPPC was 10 μ M. All analogs were tested at single 10 μ M concentrations. Absorbance of liberated *para*-nitrophenol at 405 nm was determined up to 1 hour (where responses were linear) and was normalized to vehicle control.

MM1 Hepatoma Cell Invasion of Endothelial Monolayers—The highly invasive MM1 cells originally isolated from the AH130 rat hepatoma cells were a kind gift from (Dr. Michiko Mukai, Osaka University^[36, 50]). These cells were grown in suspension in DMEM supplemented with 10% fetal bovine serum, 2mM glutamine, 100 units/ml penicillin and 10 μ g/ml streptomycin. The isolation and culture of mesothelial cells from C57BL/6 mice has been previously described elsewhere HUVECs obtained from VEC Technologies Inc., (Rensselaer, NY, USA) were grown in MCDB-131 complete medium containing 10% fetal bovine serum, 90 μ g/ml heparin, 10ng/ml EGF, 1 μ g/ml hydrocortisone, 0.2mg/ml EndoGrowth supplement, 100 units/ml penicillin G, 100 μ g/ml streptomycin and 25 μ g/ml Amphotericin B (all from VEC Technologies). Tumor cell invasion was performed by seeding 1.3×10^5 HUVECs (passage 7) into each well of a 12-well plate pre-coated with 0.2% gelatin (Sigma) and cultured for 2 days to form a confluent monolayer. Mesothelial cells harvested from two-three mice were initially plated in 6-well plates and grown to confluency in DMEM medium supplemented with 10% fetal bovine serum in the presence of 100 units/ml penicillin G and 10 μ g/ml streptomycin. When confluent, the mesothelial cells were split into three equal aliquots ($\sim 5 \times 10^4$ cells each) and plated to three wells of a 12-well plate and grown to confluence. For all invasion assays, MM1 cells were pre-stained with 2 μ g/ml calcein AM (Invitrogen, USA) for 2 h, rinsed once, and seeded at a density of

5×10^4 cells/well over the monolayers. Tumor cells were left to invade the HUVEC monolayer for 24 h in MCDB-131 complete media containing 1% serum with or without addition of 1.5 μM LPC. MM1 cells were plated on the mesothelial monolayers in 2% fetal bovine serum-supplemented DMEM medium in the presence of 100 units/ml penicillin G and 10 $\mu\text{g/ml}$ streptomycin with or without 1.5 μM LPC (5 μM for the mesothelium cells) and invasion was allowed to proceed for 20 h. The day after MM1 cell seeding, non-invaded tumor cells were removed by repeated five rinses of the HUVEC monolayer (three rinses for the mesothelial monolayers) with PBS (containing Ca^{2+} and Mg^{2+}) followed by fixation with 10% buffered formalin. The number of tumor cells that penetrated the monolayer was photographed under a NIKON TiU inverted microscope using phase-contrast and fluorescence illumination in a minimum of five non-overlapping fields at $100\times$ magnification. The fluorescent images were overlayed on top of the phase contrast images using the Elements BR software (version 3.1x) and the invaded MM1 cells showing the characteristic flattened morphology in the plane of focus underneath the monolayer were counted. For the invasion assay, LPC dissolved in chloroform was dried, re-dissolved in 1mM charcoal-stripped BSA in PBS and added immediately to the HUVEC or mesothelium monolayer and co-cultured with MM1 cells with or without the ATX inhibitors. The final BSA concentration was 30 μM .

Acute Murine Toxicity Studies

To determine acute toxicity of compound **22** we injected it at 1 – 10–30 mg/kg/day dose intraperitoneally into groups of six adult Swiss mice for 10 days and monitored behavior and weight daily. The mice were sacrificed on day 14 and necropsied for signs of organ damage.

Pharmacokinetic Studies

Studies were approved by the Institute of Animal Care and Use Committee (IACUC) of University of Tennessee, Memphis. Animal treatment was in accordance with regulations outlined in the United States Department of Agriculture (USDA) Animal Welfare Act (9CFR, Parts 1, 2, and 3) and the conditions specified in the Guide for the Care and Use of Laboratory Animals. Male Sprague-Dawley rats (250–275 grams) pre-cannulated with jugular and femoral vein catheters were obtained from Charles River Laboratories (Wilmington, MA). The patency of the jugular and femoral vein catheters was maintained every other day with heparinized glycerol solution (500 IU heparin/mL final solution in 50% glycerol) according to vendor's instructions. Animals were fed a normal laboratory diet, allowed *ad libitum* access to water and maintained on a 12-h light-dark cycle (25°C).

Compound **22** was dissolved in propylene glycol and diluted in normal rat plasma. Rats were given a single intravenous (i.v.) bolus dose of compound **22** (5.8 mg/kg) via the femoral vein catheter. Blood (200 μL) was collected from the jugular vein catheter at timed intervals 5, 15, 30, 45, 60, 120, 240, 480, and 1440 minutes, into Microtainer[®] brand tubes with lithium heparin (BD, Franklin Lakes, NJ). Samples were centrifuged (10,000 $\times$ g, 25°C for 3 minutes) and the plasma was stored at -80°C until analysis. Sample aliquots (50 μL) were mixed with 100 μL citrate phosphate buffer (30mM citric acid, 40mM Na_2HPO_4 , pH4.0), 400 μL water saturated butanol, 200ng internal standard (compound **10**), and 100ng LPA 17:0. Samples were centrifuged (10,000 $\times$ g for 1 minute) and the butanol layer was transferred to a new tube and dried down under argon gas. Plasma concentrations were determined similarly to a previously described method.^[59] Compound **22** and LPA concentrations were determined by liquid chromatography-mass spectrometry (LC-MS/MS) using an Applied Biosystems Sciex ABI 4000 QTRAP tandem mass spectrometer (Foster City, CA) equipped with a Turboionspray[™] interface, a Shimadzu LC-10ADvp HPLC pump (Columbia, MD) with a Leap HTS PAL autosampler (Carrboro, NC). Samples (5 μl) were injected onto a C8 analytical column (Symmetry, 3.5 μm , 2.1mm $\times$ 100 mm, Waters

Corporation, Milford, MA) with a solvent consisting of acetonitrile and water containing 10mM ammonium acetate using an isocratic flow rate of 0.3mL/min^{9,160]} The spectra were processed using Analyst software, version 1.5. The molecular species of LPA and compound **22** were analyzed by multiple reaction monitoring (MRM) in negative ion mode. The Q3 (product ion) was set at m/z 153.0 for LPA and 79.0 for compound **22** and 186.0 for compound **10**. Q1 was set for the neutral molecular ion. Quantification was done by calculating the ratio of peak area to that of the appropriate internal standard.

Pharmacokinetic data analysis

Compound **22** plasma concentration-time data was analyzed by non-compartmental methods. The area under the plasma concentration-time curve from time 0 to infinity ($AUC_{0-\infty}$) was calculated by the trapezoidal rule with extrapolation to time infinity. The terminal half-life ($t_{1/2}$) was calculated as $0.693/\lambda_z$, where λ_z was the terminal phase elimination constant. The plasma clearance (CL) was calculated as $CL = \text{dose}_{i.v.} / AUC_{i.v., 0-\infty}$, where $\text{dose}_{i.v.}$ and $AUC_{i.v., 0-\infty}$ were the i.v. dose and corresponding area under the curve from time 0 to infinity, respectively. The apparent volume of distribution at equilibrium (V_{dss}) was calculated as $V_{dss} = CL \cdot MRT$, where MRT was the mean residence time after the i.v. bolus dose. MRT was calculated as $MRT = (AUMC_{i.v., 0-\infty}) / (AUC_{i.v., 0-\infty})$, where $AUMC_{i.v., 0-\infty}$ was the area under the first moment of the plasma concentration-time curve extrapolated to infinity.

B16-F10 Murine Melanoma Metastasis Model—All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Tennessee and the M.D. Anderson Cancer Center and were consistent with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication 85–23, revised 1985). Eight-week-old female C57Bl/6 mice were injected with 5×10^4 cells/animal via tail veins and divided randomly into 4 groups. Each group then received selective ATX inhibitor (**22** or **30b**) or dual ATX and LPA receptor antagonist LPA-BP^[35] all at 0.5 mg/kg/injection or vehicle (PBS with 1% DMSO) via intraperitoneal injection 30 min after the B16-F10 injection and daily for an additional 10 days. Subsequently, animals in all groups were monitored for another 10 days without treatment. At day 21, all mice were sacrificed and lungs were harvested, inflated, and fixed with 10% formalin. The number of metastatic nodules on the lung surface was counted. The number of lung nodules was compared to vehicle treated group by one-way ANOVA followed by Newman-Keuls multiple comparison test and $p < 0.05$ was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by CA 92160 (G.T.), the Van Vleet Oncology Research Fund (G.T.), the Breast Cancer Research Foundation (N.P.) and Lpath Inc. (G.M.).

Reference List

1. Umezū-Goto M, Kishi Y, Taira A, Hama K, Dohmae N, Takio K, Yamori T, Mills GB, Inoue K, Aoki J, Arai H. J Cell Biol. 2002; 158(2):227–233. [PubMed: 12119361]
2. Tokumura A, Majima E, Kariya Y, Tominaga K, Kogure K, Yasuda K, Fukuzawa K. J Biol Chem. 2002; 277(42):39436–39442. [PubMed: 12176993]
3. Wu JM, Xu Y, Skill NJ, Sheng H, Zhao Z, Yu M, Saxena R, Maluccio MA. Mol Cancer. 2010; 9(1): 71. [PubMed: 20356387]

4. Nouh MA, Wu XX, Okazoe H, Tsunemori H, Haba R, Abou-Zeid AM, Saleem MD, Inui M, Sugimoto M, Aoki J, Kakehi Y. *Cancer Sci.* 2009; 100(9):1631–1638. [PubMed: 19549252]
5. Yang Y, Mou L, Liu N, Tsao MS. *Am J Respir Cell Mol Biol.* 1999; 21(2):216–222. [PubMed: 10423404]
6. Samadi N, Gaetano C, Goping IS, Brindley DN. *Oncogene.* 2009; 28(7):1028–1039. [PubMed: 19079345]
7. Vidot S, Witham J, Agarwal R, Greenhough S, Bamrah HS, Tigyi GJ, Kaye SB, Richardson A. *Cell Signal.* 2010; 22(6):926–935. [PubMed: 20100569]
8. Masuda A, Nakamura K, Izutsu K, Igarashi K, Ohkawa R, Jona M, Higashi K, Yokota H, Okudaira S, Kishimoto T, Watanabe T, Koike Y, Ikeda H, Kozai Y, Kurokawa M, Aoki J, Yatomi Y. *Br J Haematol.* 2008; 143(1):60–70. [PubMed: 18710386]
9. Gaetano CG, Samadi N, Tomsig JL, Macdonald TL, Lynch KR, Brindley DN. *Mol Carcinog.* 2009; 48(9):801–809. [PubMed: 19204929]
10. Kehlen A, Englert N, Seifert A, Klonisch T, Dralle H, Langner J, Hoang-Vu C. *Int J Cancer.* 2004; 109(6):833–838. [PubMed: 15027116]
11. Liu S, Umez-Goto M, Murph M, Lu Y, Liu W, Zhang F, Yu S, Stephens LC, Cui X, Murrow G, Coombes K, Muller W, Hung MC, Perou CM, Lee AV, Fang X, Mills GB. *Cancer Cell.* 2009; 15(6):539–550. [PubMed: 19477432]
12. Gugger M, White R, Song S, Waser B, Cescato R, Riviere P, Reubi JC. *Dis Markers.* 2008; 24(1):41–50. [PubMed: 18057535]
13. Sakagami H, Aoki J, Natori Y, Nishikawa K, Kakehi Y, Natori Y, Arai H. *J Biol Chem.* 2005; 280(24):23084–23093. [PubMed: 15788404]
14. Duan RD, Bergman T, Xu N, Wu J, Cheng Y, Duan J, Nelander S, Palmberg C, Nilsson A. *J Biol Chem.* 2003; 278(40):38528–38536. [PubMed: 12885774]
15. Wu J, Nilsson A, Jonsson BA, Stenstad H, Agace W, Cheng Y, Duan RD. *Biochem J.* 2006; 394(Pt 1):299–308. [PubMed: 16255717]
16. Tanaka M, Okudaira S, Kishi Y, Ohkawa R, Iseki S, Ota M, Noji S, Yatomi Y, Aoki J, Arai H. *J Biol Chem.* 2006; 281(35):25822–25830. [PubMed: 16829511]
17. van Meeteren LA, Ruurs P, Stortelers C, Bouwman P, van Rooijen MA, Pradere JP, Pettit TR, Wakelam MJ, Saulnier-Blache JS, Mummery CL, Moolenaar WH, Jonkers J. *Mol Cell Biol.* 2006; 26(13):5015–5022. [PubMed: 16782887]
18. Fotopoulou S, Oikonomou N, Grigorieva E, Nikitopoulou I, Paparountas T, Thanassopoulou A, Zhao Z, Xu Y, Kontoyiannis DL, Remboutsika E, Aidinis V. *Dev Biol.* 2010; 339(2):451–464. [PubMed: 20079728]
19. Baker DL, Morrison P, Miller B, Riely CA, Tolley B, Westermann AM, Bonfrer JM, Bais E, Moolenaar WH, Tigyi G. *JAMA.* 2002; 287(23):3081–3082. [PubMed: 12069669]
20. Sutphen R, Xu Y, Wilbanks GD, Fiorica J, Grendys EC Jr, LaPolla JP, Arango H, Hoffman MS, Martino M, Wakeley K, Griffin D, Blanco RW, Cantor AB, Xiao YJ, Krischer JP. *Cancer Epidemiol Biomarkers Prev.* 2004; 13(7):1185–1191. [PubMed: 15247129]
21. Xu Y, Gaudette DC, Boynton JD, Frankel A, Fang XJ, Sharma A, Hurteau J, Casey G, Goodbody A, Mellors A. *Clin Cancer Res.* 1995; 1(10):1223–1232. [PubMed: 9815916]
22. Dimova I, Raitcheva S, Dimitrov R, Doganov N, Toncheva D. *Eur J Cancer.* 2006; 42(5):674–679. [PubMed: 16458500]
23. Euer N, Schwirzke M, Evtimova V, Burtcher H, Jarsch M, Tarin D, Weidle UH. *Anticancer Res.* 2002; 22(2A):733–740. [PubMed: 12014644]
24. Liu S, Umez-Goto M, Murph M, Lu Y, Liu W, Zhang F, Yu S, Stephens LC, Cui X, Murrow G, Coombes K, Muller W, Hung MC, Perou CM, Lee AV, Fang X, Mills GB. *Cancer Cell.* 2009; 15(6):539–550. [PubMed: 19477432]
25. ES, Lai YJ, Tsukahara R, Chen CS, Fujiwara Y, Yue J, Yu JH, Guo H, Kihara A, Tigyi G, Lin FT. *J Biol Chem.* 2009; 284(21):14558–14571. [PubMed: 19293149]
26. Jazaeri AA, Awtrey CS, Chandramouli GV, Chuang YE, Khan J, Sotiriou C, Aprelikova O, Yee CJ, Zorn KK, Birrer MJ, Barrett JC, Boyd J. *Clin Cancer Res.* 2005; 11(17):6300–6310. [PubMed: 16144934]

27. Tsuda S, Okudaira S, Moriya-Ito K, Shimamoto C, Tanaka M, Aoki J, Arai H, Murakami-Murofushi K, Kobayashi T. *J Biol Chem.* 2006; 281(36):26081–26088. [PubMed: 16837466]
28. van Meeteren LA, Ruurs P, Christodoulou E, Goding JW, Takakusa H, Kikuchi K, Perrakis A, Nagano T, Moolenaar WH. *J Biol Chem.* 2005; 280(22):21155–21161. [PubMed: 15769751]
29. Durgam GG, Tsukahara R, Makarova N, Walker MD, Fujiwara Y, Pigg KR, Baker DL, Sardar VM, Parrill AL, Tigyi G, Miller DD. *Bioorg Med Chem Lett.* 2006; 16(3):633–640. [PubMed: 16263282]
30. Albers HM, van Meeteren LA, Egan DA, van Tilburg EW, Moolenaar WH, Ovaa H. *J Med Chem.* 2010; 53(13):4958–4967. [PubMed: 20536182]
31. Albers HM, Dong A, van Meeteren LA, Egan DA, Sunkara M, van Tilburg EW, Schuurman K, van TO, Morris AJ, Smyth SS, Moolenaar WH, Ovaa H. *Proc Natl Acad Sci U S A.* 2010; 107(16):7257–7262. [PubMed: 20360563]
32. Baker DL, Fujiwara Y, Pigg KR, Tsukahara R, Kobayashi S, Murofushi H, Uchiyama A, Murakami-Murofushi K, Koh E, Bandle RW, Byun HS, Bittman R, Fan D, Murph M, Mills GB, Tigyi G. *J Biol Chem.* 2006; 281(32):22786–22793. [PubMed: 16782709]
33. Gajewiak J, Tsukahara R, Fujiwara Y, Tigyi G, Prestwich GD. *Org Lett.* 2008; 10(6):1111–1114. [PubMed: 18284246]
34. Gajewiak J, Tsukahara R, Tsukahara T, Fujiwara Y, Yu S, Lu Y, Murph M, Mills GB, Tigyi G, Prestwich GD. *ChemMedChem.* 2007; 2(12):1789–1798. [PubMed: 17952880]
35. Zhang H, Xu X, Gajewiak J, Tsukahara R, Fujiwara Y, Liu J, Fells JI, Perygin D, Parrill AL, Tigyi G, Prestwich GD. *Cancer Res.* 2009; 69(13):5441–5449. [PubMed: 19509223]
36. Uchiyama A, Mukai M, Fujiwara Y, Kobayashi S, Kawai N, Murofushi H, Inoue M, Enoki S, Tanaka Y, Niki T, Kobayashi T, Tigyi G, Murakami-Murofushi K. *Biochim Biophys Acta.* 2007; 1771(1):103–112. [PubMed: 17123862]
37. Hoeglund AB, Bostic HE, Howard AL, Wanjala IW, Best MD, Baker DL, Parrill AL. *J Med Chem.* 2010; 53(3):1056–1066. [PubMed: 20041668]
38. Hoeglund AB, Howard AL, Wanjala IW, Pham TC, Parrill AL, Baker DL. *Bioorg Med Chem.* 2010; 18(2):769–776. [PubMed: 20005724]
39. North EJ, Osborne DA, Bridson PK, Baker DL, Parrill AL. *Bioorg Med Chem.* 2009; 17(9):3433–3442. [PubMed: 19345587]
40. North EJ, Howard AL, Wanjala IW, Pham TC, Baker DL, Parrill AL. *J Med Chem.* 2010; 53(8):3095–3105. [PubMed: 20349977]
41. Parrill AL. *Biochim Biophys Acta.* 2008; 1781(9):540–546. [PubMed: 18501204]
42. Parrill AL, Baker DL. *Anticancer Agents Med Chem.* 2008; 8(8):917–923. [PubMed: 19075574]
43. Ferry G, Moulharat N, Pradere JP, Desos P, Try A, Genton A, Giganti A, Beucher-Gaudin M, Lonchampt M, Bertrand M, Saulnier-Blache JS, Tucker GC, Cordi A, Boutin JA. *J Pharmacol Exp Ther.* 2008; 327(3):809–819. [PubMed: 18755937]
44. Kim YC, Brown SG, Harden TK, Boyer JL, Dubyak G, King BF, Burnstock G, Jacobson KA. *J Med Chem.* 2001; 44(3):340–349. [PubMed: 11462975]
45. Jiang G, Xu Y, Fujiwara Y, Tsukahara T, Tsukahara R, Gajewiak J, Tigyi G, Prestwich GD. *ChemMedChem.* 2007; 2(5):679–690. [PubMed: 17443831]
46. Valentine WJ, Kiss GN, Liu J, ES, Gotoh M, Murakami-Murofushi K, Pham TC, Baker DL, Parrill AL, Lu X, Sun C, Bittman R, Pyne NJ, Tigyi G. *Cell Signal.* 2010; 22(10):1543–1553. [PubMed: 20566326]
47. Williams JR, Khandoga AL, Goyal P, Fells JI, Perygin DH, Siess W, Parrill AL, Tigyi G, Fujiwara Y. *J Biol Chem.* 2009; 284(25):17304–17319. [PubMed: 19366702]
48. Imamura F, Horai T, Mukai M, Shinkai K, Akedo H. *Jpn J Cancer Res.* 1991; 82(5):493–496. [PubMed: 1905695]
49. Imamura F, Horai T, Mukai M, Shinkai K, Sawada M, Akedo H. *Biochem Biophys Res Commun.* 1993; 193(2):497–503. [PubMed: 8390242]
50. Mukai M, Imamura F, Ayaki M, Shinkai K, Iwasaki T, Murakami-Murofushi K, Murofushi H, Kobayashi S, Yamamoto T, Nakamura H, Akedo H. *Int J Cancer.* 1999; 81(6):918–922. [PubMed: 10362139]

51. Mukai M, Nakamura H, Tatsuta M, Iwasaki T, Togawa A, Imamura F, Akedo H. *FEBS Lett.* 2000; 484(2):69–73. [PubMed: 11068034]
52. Watanabe N, Ikeda H, Nakamura K, Ohkawa R, Kume Y, Tomiya T, Tejima K, Nishikawa T, Arai M, Yanase M, Aoki J, Arai H, Omata M, Fujiwara K, Yatomi Y. *Life Sci.* 2007; 81(12):1009–1015. [PubMed: 17850827]
53. Watanabe N, Ikeda H, Nakamura K, Ohkawa R, Kume Y, Aoki J, Hama K, Okudaira S, Tanaka M, Tomiya T, Yanase M, Tejima K, Nishikawa T, Arai M, Arai H, Omata M, Fujiwara K, Yatomi Y. *J Clin Gastroenterol.* 2007; 41(6):616–623. [PubMed: 17577119]
54. Altman MK, Gopal V, Jia W, Yu S, Hall H, Mills GB, McGinnis AC, Bartlett MG, Jiang G, Madan D, Prestwich GD, Xu Y, Davies MA, Murph MM. *Mol Cancer.* 2010; 9:140. [PubMed: 20529378]
55. Gierse J, Thorarensen A, Beltey K, Bradshaw-Pierce E, Cortes-Burgos L, Hall T, Johnston A, Murphy M, Nemirovskiy O, Ogawa S, Pegg L, Pelc M, Prinsen M, Schnute M, Wendling J, Wene S, Weinberg R, Wittwer A, Zweifel B, Masferrer J. *J Pharmacol Exp Ther.* 2010; 334(1):310–317. [PubMed: 20392816]
56. Prestwich GD, Xu Y, Qian L, Gajewiak J, Jiang G. *Biochem Soc Trans.* 2005; 33(Pt 6):1357–1361. [PubMed: 16246118]
57. Jansen S, Andries M, Vekemans K, Vanbilloen H, Verbruggen A, Bollen M. *Cancer Lett.* 2009; 284(2):216–221. [PubMed: 19482419]
58. Durgam GG, Virag T, Walker MD, Tsukahara R, Yasuda S, Liliom K, van Meeteren LA, Moolenaar WH, Wilke N, Siess W, Tigyi G, Miller DD. *J Med Chem.* 2005; 48(15):4919–4930. [PubMed: 16033271]
59. Kosanam H, Ma F, He H, Ramagiri S, Gududuru V, Tigyi GJ, Van RK, Miller DD, Yates CR. *J Chromatogr B Analyt Technol Biomed Life Sci.* 2010; 878(26):2379–2383.
60. Tokumura A, Carbone LD, Yoshioka Y, Morishige J, Kikuchi M, Postlethwaite A, Watsky MA. *Int J Med Sci.* 2009; 6(4):168–176. [PubMed: 19521548]

Figure 1.
ATX inhibitor S32826

Figure 2.

Inhibition of ATX-mediated hydrolysis of FS3 by LPA, S32826, compounds **22**, or **30b**. ATX (2nM) and 1 μ M FS3 were incubated in the presence of increasing concentrations of the inhibitors for 2h and the fluorescent product was measured and expressed as per cent of FS3 hydrolysis by ATX in the presence of vehicle less the autolysis of FS3 ($n=3 \pm$ SD).

Figure 3. Inhibition of LPA production and pNP-TMP hydrolysis by compounds **22** and **30b**. Panel A: A representative TLC image shows that compounds **22** and **30b** applied at 3 μ M inhibited the hydrolysis of 0.5 μ M ADMAN-LPC by 100 nM ATX. Panel B. Compound **22** and **30b** applied at 10 μ M inhibit the hydrolysis of 5 mM pNP-TMP by 4 nM ATX more than the product LPA 18:1 applied at 10 μ M.

Figure 4. Profiling of LPA receptor and ATX transcripts in MM1 (panel A), mouse mesothelial (panel B) and HUVEC cells (panel C) using quantitative real-time PCR. (Panel D) LPC dose-dependently increases the invasion of mesothelial monolayers by MM1 cells. (Panel E) Compounds **22** and **30b** dose dependently inhibit the invasion of mesothelial monolayers induced by LPC (1.5 μM) that is reduced to the level of control at and above 3 μM concentration. (Panel F) Compounds **22** and **30b** block LPC-induced but do not alter the basal rate of invasion of HUVEC

Figure 5.

Plasma level of compound **22** (panel A) and two LPA species (panel B) after i.v. administration with a single dose of 5.8 mg/kg. Note that compound **22** has a relatively long half-life calculated at 10.5 h, plasma mean residence time 456 ± 33 min and LPA 18:2 and 20:4 remain depressed over the 24 h duration of the study.

Figure 6.
Effect of compound **22**, **30b**, and LPA -BP on the lung metastasis of B16-F10 melanoma. Panel A – Representative lungs taken from the treatment groups. Panel B – Lung nodule counts in the treatment groups. * Denotes $p < 0.05$ relative to vehicle.

Scheme 1.

Reagents and conditions: (a) *N,N*-diisopropylethylamine, THF, Reflux, 24 h; (b) TMSBr, CH₃CN, Reflux, 1 h; (c) MeOH, 30 min, RT.

Scheme 2.

Reagents and conditions: (a) K_2CO_3 , 18-crown-6, acetone, Reflux, 16 h; (b) PBr_3 , ether, RT, 30 min; (c) $P(OMe)_3$, Reflux, 18 h; (d) $TMSBr$, CH_3CN , Reflux, 1 h; (e) $MeOH$, 30 min, RT.

Scheme 3.

Reagents and conditions: (a) Pd(OAc)₂, Et₃N, DMF, Reflux, 16 h; (b) LAH, THF, 0 °C-RT, 4 h; (c) H₂/Pd/C, MeOH; (d) PBr₃, ether, RT, 30 min; (e) P(OMe)₃, Reflux, 18h; (f) TMSBr, CH₃CN, Reflux, 1 h; (g) MeOH, 30 min, RT.

Scheme 4.

Reagents and conditions: (a) Pd(OAc)₂, Et₃N, DMF, Reflux, 16 h; (b) LAH, THF, 0 °C-RT, 4 h; (c) H₂/Pd/C, MeOH; (d) PBr₃, ether, RT, 30 min; (e) P(OMe)₃, Reflux, 18h; (f) TMSBr, CH₃CN, Reflux, 1 h; (g) MeOH, 30 min, RT.

Scheme 5.

Reagents and conditions: (a) PDC, CH₂Cl₂, RT, 16 h; (b) HP(O)(OCH₃)₂, Et₃N, 0 °C-RT, 4 h; (c) TMSBr, CH₃CN, Reflux, 1h; (d) MeOH, 30 min, RT; (e) DAST, ether, 0 °C-RT, 1 h; (f) SOCl₂, CH₂Cl₂, Reflux, 1 h; (g) PBr₃, ether, 30 min, RT.

Scheme 6.

Reagents and conditions: (a) (i) bis(2-cyanoethyl)-*N,N*-diisopropylphosphoramidite, 1*H*-tetrazole, CH₂Cl₂, RT; (ii) 50% H₂O₂; (b) (i) bis(2-cyanoethyl)-*N,N*-diisopropylphosphoramidite, 1*H*-tetrazole, CH₂Cl₂, RT; (ii) Sulfur, Reflux, 2 h; (c) (i) KOH, MeOH; (ii) dil HCl.

Table Ia

Characterization of benzyl phosphonic acids at ATX, NPP6 and NPP7.

Compound	X	Y	R	ATX					NPP6	NPP7
				Activity, % control (10 μ M)	IC ₅₀ (μ M)	Mechanism of Inhibition	K _i (μ M)	K _i ' (μ M)		
S32826	O	CH ₂		2.6	ND	ND	N/A	N/A	102.1	101.2
4	O	CH ₂		24.1	ND	ND	N/A	N/A	102.0	93.5
10	O	CH ₂		30.4	ND	ND	N/A	N/A	102.0	100.6

Compound	X	Y	R	ATX					NPP6	NPP7
				Activity, % control (10 μ M)	IC ₅₀ (μ M)	Mechanism of Inhibition	K _i (μ M)	K _i ' (μ M)		
18	O	CH ₂		18.8	ND	ND	N/A	N/A	104.0	97.7
22	O	CH ₂		5.2	0.17	Mixed	0.27	0.28	96.5	96.7
37	O	CHOH		9.2	0.73	Mixed	0.45	0.70	99.6	101.0
41	O	CHF		42.3	17.9	Mixed	4.97	5.54	101.1	102.6
42	O	CHCl		117.9	ND	ND	N/A	N/A	100.6	99.9
43	O	CHBr		60.1	10.1	Mixed	6.10	2.97	99.3	99.5
45	O	O-CH ₂		85.2	ND	ND	N/A	N/A	100.9	98.8
47	S	O-CH ₂		15.2	1.54	Mixed	4.45	4.43	98.8	92.9

ND – not determined, NA – not applicable since the mechanism of inhibition was not determined.

Table Ib

Characterization of naphthylphosphonic acids at ATX, NPP6 and NPP7.

Compound	X	Y	R	ATX					NPP6	NPP7
				Activity, % control (10 μ M)	IC ₅₀ (μ M)	Mechanism of Inhibition	K _i (μ M)	K _i ' (μ M)		
30a	O	CH ₂		61.3	ND	ND	N/A	N/A	103.1	100.0
30b	O	CH ₂		17.3	1.40	MIXED	1.50	1.01	104.1	96.5
34a	O	CH ₂		19.9	ND	ND	N/A	N/A	101.0	98.8
34b	O	CH ₂		50.8	ND	ND	N/A	N/A	100.0	94.7

ND – not determined, NA – not applicable since the mechanism of inhibition was not determined.

Table II

COMPOUND	RH7777 VECTOR E _{MAX} 10 _{μM} (%OF ATP)	LPA ₁ (E _{MAX} 10 _{μM})	LPA ₂ (I _{MAX} 10 _{μM})	LPA ₃ (E _{MAX} 10 _{μM})	CHO VECTOR E _{MAX} 10 _{μM} (%OF ATP)	LPA ₄	B103 VECTOR E _{MAX} 10 _{μM} (%OF ATP)	LPA ₅	
								(E _{MAX} 10 _{μM})	(I _{MAX} 10 _{μM})
22	> 1	NE	23	22	> 1	NE	> 3	NE	NE
30b	> 6	NE	NE	33	> 1	NE	> 3	17	NE
37	> 1	NE	28	NE	NE	NE	NE	NE	51
41	> 3	NE	26	17	> 5	NE	> 1	NE	NE
43	> 5	NE	NE	NE	> 5	NE	> 2	NE	NE
42	> 15	ND	ND	ND	> 74	ND	> 32	ND	ND
45	> 6	NE	50	13	> 14	NE	> 2	100 (EC ₅₀ =7.9 μM)	NE
47	> 7	10	NE	44	> 14	NE	> 3	100 (EC ₅₀ =2.9 μM)	NE

NE – no effect, ND – not definable due to effect on vector transfected cells, E_{MAX} 10_{μM} – maximal response at 10 μM relative to the maximal response to either ATP in vector transfected cells or to LPA in LPA receptor expressing cells, I_{MAX} 10_{μM} – maximal inhibition of the ~EC₇₅ LPA response caused by the compound applied at 10 μM