Published in final edited form as:

Bioorg Med Chem. 2015 September 1; 23(17): 5999–6013. doi:10.1016/j.bmc.2015.06.054.

Vinyl Sulfone Analogs of Lysophosphatidylcholine Irreversibly Inhibit Autotaxin and Prevent Angiogenesis in Melanoma

Mandi M. Murph^{1,5,*}, Guowei W. Jiang^{2,*}, Molly K. Altman¹, Wei Jia¹, Duy T. Nguyen¹, Jada M. Fambrough¹, William J. Hardman³, Ha T. Nguyen¹, Sterling K. Tran¹, Ali A. Alshamrani¹, Damian Madan⁴, Jianxing Zhang², and Glenn D. Prestwich^{2,5}

¹Department of Pharmaceutical and Biomedical Sciences, The University of Georgia, College of Pharmacy, 240 W. Green Street, Athens, Georgia 30602

²Department of Medicinal Chemistry, The University of Utah, 419 Wakara Way, Suite 205, Salt Lake City, Utah 84108-1257

³The University of Georgia and Georgia Regents University Medical Partnership, 1425 Prince Avenue, Athens, Georgia 30606

⁴Echelon Biosciences Incorporated, 675 Arapen Way, Suite 302, Salt Lake City, Utah 84108

Abstract

Autotaxin (ATX) is an enzyme discovered in the conditioned medium of cultured melanoma cells and identified as a protein that strongly stimulates motility. This unique ectonucleotide pyrophosphatase and phosphodiesterase facilitates the removal of a choline headgroup from lysophosphatidylcholine (LPC) to yield lysophosphatidic acid (LPA), which is a potent lipid stimulator of tumorigenesis. Thus, ATX has received renewed attention because it has a prominent role in malignant progression with significant translational potential. Specifically, we sought to develop active site-targeted irreversible inhibitors as anti-cancer agents. Herein we describe the synthesis and biological activity of an LPC-mimetic electrophilic affinity label that targets the active site of ATX, which has a critical threonine residue that acts as a nucleophile in the lysophospholipase D reaction to liberate choline. We synthesized a set of quaternary ammonium derivative-containing vinyl sulfone analogs of LPC that function as irreversible inhibitors of ATX and inactivate the enzyme. The analogs were tested in cell viability assays using multiple cancer cell lines. The IC₅₀ values ranged from 6.74 – 0.39 μ M, consistent with a K_i of 3.50 μ M for inhibition of ATX by the C₁₆H₃₃ vinyl sulfone analog CVS-16 (**10b**). A phenyl vinyl sulfone control compound, PVS-16, lacking the choline-like quaternary ammonium mimicking head group moiety, had little effect on cell viability and did not inhibit ATX. Most importantly, CVS-16 (**10b**) significantly inhibited melanoma progression in an *in vivo* tumor model by preventing angiogenesis. Taken together, this suggests that CVS-16 (**10b**) is a potent and irreversible ATX inhibitor with significant biological activity both *in vitro* and *in vivo*.

⁵Corresponding authors; mmurph@uga.edu, gprestwich@pharm.utah.edu.

*These authors contributed equally to this research study.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Abstract

Keywords

angiogenesis; anticancer therapy; autotaxin; lysophosphatidylcholine; melanoma; vinyl sulfone

INTRODUCTION

The ectonucleotide pyrophosphatase/phosphodiesterase 2 or its more common designation autotaxin (ATX), is an enzyme with lysophospholipase D activity that converts lysophosphatidylcholine (LPC) to lysophosphatidic acid (LPA) ¹ by hydrolysis of the choline head group from LPC. Although it is central to the biosynthesis of LPA, ATX also hydrolyzes p-nitrophenyl thymidine-5'-monophosphate, which is a type I phosphodiesterase substrate ². Thus, it is a multi-faceted enzyme with more than one important function.

ATX was isolated from conditioned medium of melanoma cells ³, and was discovered to play a major role in the development of both the vascular ⁴ and nervous systems ⁵ as well as malignancy ⁶. In particular, ATX promotes cancer progression, since it can increase growth factor signaling, cell survival, proliferation and migration in many cancers, including pancreatic cancer ⁷, cutaneous and uveal melanoma ⁸, breast cancer ^{6b}, follicular lymphoma ⁹, glioblastoma multiforme ¹⁰ and gynecologic malignancies ¹¹. In addition, ATX also protects cancer cells from chemotherapy-induced apoptosis ¹², which could significantly compromise cancer treatment and result in worsened outcomes when ATX is present in the tumor microenvironment.

Another occurrence affecting cancer treatment is angiogenesis. This is the process whereby tumors develop blood vessels to access nutrients and oxygen from circulation and is necessary for tumor growth beyond 1 mm, at which point necrosis occurs in otherwise hypoxic tissues. To state succinctly – angiogenesis is required for tumor progression. Without angiogenesis, the growing tumor will not thrive and will remain small. Because of this dependency, anticancer therapeutics targeting angiogenesis exploit tumors by inhibiting growth factors required for the formation of new blood vessels, such as the vascular endothelial growth factor (VEGF). This and other growth factors are secreted by the tumor when it exceeds a certain distance from its primary blood supply and senses hypoxia. On the other hand, since the circulatory system is exploited by intravenous anticancer therapeutics to reach the insides of tumors, timing is critical to properly treat patients with angiogenesis inhibitors.

Interestingly, ATX is a direct and indirect angiogenic factor that stimulates human endothelial cells to form tubules and tumors to become more hyperemic¹³. It is thus not surprising that the outcome of knocking out ATX on vasculogenesis results in embryonic lethal mutations in mice embryos which display aberrant blood vessel formation upon death⁴. Mechanistically, an increase in VEGF increases ATX expression and secretion among (at least) ovarian cancer cells. This resultant increase in ATX then results in more lysophosphatidic acid and also drives cells to produce more of the receptors LPA3, LPA4 and VEGFR2¹⁴. This represents a positive feedback loop between ATX and growth factors involved in angiogenesis, especially since lysophosphatidic acid also stimulates VEGF production. In other words, ATX plays a central role in angiogenesis and thus, tumor progression.

Because of the critical role ATX has in angiogenesis and various malignancies, extensive research is devoted to the design, synthesis and evaluation of novel inhibitors of ATX^{8a, 8b, 15} as well as the evaluation of natural substances with inhibitory activity¹⁶. Some recently designed chemical inhibitors have not possessed the necessary characteristics to proceed into clinical development due to a lack of bioavailability or large millimolar concentrations required for activity. Interestingly, ATX is product-inhibited by both sphingosine 1-phosphate and lysophosphatidic acid¹⁷. This suggests that these bioactive lipids are capable of regulating their own synthesis in the microenvironment and this knowledge bestows a scheme to chemically exploit their abundance. Other studies have elucidated the crystal structure of ATX¹⁸ and further described how the enzyme discriminates substrates¹⁹, which resulted in novel ideas for the design of additional ATX inhibitors.

Herein we report the synthesis and biological testing of a series of alkyl vinyl sulfone analogs of LPC. These analogs feature a quaternary ammonium derivative, a reactive vinyl sulfone, and alkyl chains varying in length from C₆ to C₁₈. We also prepared a control compound, a phenyl vinyl sulfone, which lacked the targeting by the quaternary ammonium derivative but retained the long alkyl chain. Vinyl sulfone derivatives are widely known as irreversible inhibitors of cysteine proteases²⁰. The mechanism of this irreversible inhibition depends on the vinyl sulfone acting as a Michael acceptor covalently reacting with the soft nucleophiles, such as thiols²¹. Studies indicate that peptide vinyl sulfone inhibitors also modify the active site threonine (Thr) of the *Escherichia coli* HsIV homologue and *Rhodococcus* proteasome^{21b, 22}. Similar to the inhibition of cysteine protease, the inhibition of proteasome by peptide vinyl sulfone also involves covalent modification of the *N*-terminal Thr of the catalytic β subunits, *via* a Michael addition²¹. Taken together, this suggested that vinyl sulfone might be an appropriate electrophile for targeting the active site Thr of ATX.

We hypothesized that the choline headgroup of ATX was an important recognition element for ATX, and would position the vinyl sulfone moiety in the active site in the vicinity of the Thr residue. A straight chain alkyl group was positioned in the 2-position of the vinyl sulfone, leaving a reactive, unsubstituted 1-position available to interact with an active site nucleophile. Several alkyl chains varying in length from C₆ to C₁₈ were employed to identify the optimal analog. We proposed that the metal-activated hydroxyl group of the

catalytic-site threonine could attack the vinyl sulfone and form a covalent bond that would irreversibly inactivate the enzyme.

Indeed, we observed that vinyl sulfone analogs could reduce *in vitro* cell viability, cell motility, wound closure and melanoma growth *in vivo*. We previously synthesized ATX inhibitors and examined their biological activity against melanoma^{8a, 8b, 23} and other malignancies^{15d, 15e, 24}. Since the *in vitro* activity observed by ATX inhibitors on melanoma cells is striking, we hypothesized that the mechanism of action is a directly-targeted, cellular consequence. However, we did not observe a direct effect against proliferation of melanoma models *in vivo*. Instead, the animal tumors treated with the highest compound concentration displayed significantly smaller tumors than controls, which resulted from an inhibition of angiogenesis, not mitogenesis. Herein we report the synthesis of novel vinyl sulfones and their action against the progression of melanoma via angiogenesis inhibition.

MATERIALS AND METHODS

General chemicals and spectroscopy

All synthetic reagent chemicals and solvents were purchased from Aldrich, Alfa Aesar or Acros Chemical Corporation and used without prior purification. Thin-layer chromatography was performed on pre-coated silica gel aluminum sheets (EM SCIENCE silica gel 60F₂₅₄). Purification of compounds was carried out by normal phase chromatography (ISCO Combiflash, Silica gel 230~400 mesh). NMR spectra were recorded using a Varian Mercury 400 at 400 MHz (¹H), 101 MHz (¹³C), 162 MHz (³¹P), 376 MHz (¹⁹F) at 25°C. Chemical shifts are given in ppm with TMS as internal standard (δ = 0.00); ³¹P, 85% H₃PO₄ (δ = 0.00). NMR peaks were assigned by MestRe-C (4.9.9.9). Low resolution mass spectra were obtained on HP5971A MSD double focusing mass spectrometer instrument. For biological studies, oleoyl (18:1) lysophosphatidic acid was purchased from Avanti Polar Lipids (Alabaster, AL) and reconstituted in chloroform and methanol. Prior to cell incubation, organic solutions were removed and ATX was added in 0.1% charcoal-stripped BSA.

Chemical synthesis of intermediates and final products

Ethyl 2-bromopropionate **2** (5 g, 36.0 mmol) was dissolved in acetone (200 mL), K₂CO₃ (7.45 g, 54.0 mmol, 1.5 equiv.) was added, followed by of methyl-2 mercaptoacetate **1** (4.2 g, 39.5 mmol, 1.1 equiv.) and heated at reflux for 2 h. After cooling to room temperature the mixture was filtered out, and then the white cake was washed with acetone (25 mL $\times$ 2). All combined organic solvents were evaporated under reduced pressure. The residue was purified on silica column (ethyl acetate-hexanes, 15 to 40%) to yield the pure product methyl 2-(3-hydroxypropylthio)acetate **3** (5.78 g, 35.3 mmol, 98%).

¹H NMR (CDCl₃) δ 3.67 (s, 3H), 3.66 (t, *J* = 6.0 Hz, 2H), 3.19 (s, 2H), 2.68 (t, *J* = 7.2 Hz, 2H), 2.54 (s, 1H), 1.78 (m, 2H); **¹³C NMR** (CDCl₃) δ 171.1 (s), 61.0 (s), 52.4 (s), 33.4 (s), 31.5 (s), 29.2 (s); **MS** (ESI) *m/z* 165.2 (M⁺+1).

Trityl chloride (3.34g, 12mmol), triethylamine (2.5mL, 25mmol) and the alcohol **3** (1.64g, 10 mmol) was added to CH₂Cl₂ (100mL). The reaction mixture was stirred at room temperature (rt) overnight. The reaction mixture was then washed twice with 5% NaHCO₃ (60 mL), followed by saturated NaCl solution (50 mL). The organic layer was dried over Na₂SO₄, solvents were removed by evaporation, and the residue was purified on silica column (ethyl acetate-hexanes, 0 to 10%) to yield the pure product, methyl 2-(3-(trityloxy)propylthio)acetate **4** (3.82 g, 9.4 mmol, 94%).

¹H NMR (CDCl₃) δ 7.41-7.45 (m, 6H), 7.20-7.33 (m, 9H), 3.71 (s, 3H), 3.20 (s, 2H), 3.16 (t, *J* = 6.0 Hz, 2H), 2.76 (t, *J* = 7.2 Hz, 2H), 1.89 (m, 2H); **¹³C NMR** (CDCl₃) δ 171.0 (s), 144.2 (s), 128.6 (s), 127.8 (s), 126.9 (s), 86.5 (s), 62.0 (s), 52.4 (s), 33.4 (s), 29.7 (d); **MS** (ESI) *m/z* 429.3 (M+Na⁺).

The sulfide **4** (24.2 g, 59.7 mmol, 1 equiv.) was dissolved in CH₂Cl₂ (300 mL) and *m*-CPBA (25.7 g, 149 mmol, 2.5 equiv.) was added at 0 °C. The reaction mixture was allowed warm up to rt and stirred for 8 h at rt. Volatiles were evaporated under reduced pressure. The residue was taken up in ethyl acetate (200 mL) and washed with 10% Na₂SO₃, saturated NaHCO₃, and brine. The organic layer was then dried over anhydrous Na₂SO₄. The volatiles were evaporated under reduced pressure to yield the crude mixture that was purified on silica column (ethyl acetate-hexanes, 0 to 50%) to yield the pure product, methyl 2-(3-(trityloxy)propylsulfonyl)acetate **5** (24.8 g, 56.7 mmol, 95%).

¹H NMR (CDCl₃) δ 7.41-7.51 (m, 6H), 7.20-7.38 (m, 9H), 3.97 (s, 2H), 3.81 (s, 3H), 3.38-3.46 (m, 2H), 2.29 (t, *J* = 5.6 Hz, 2H), 3.20 (s, 2H), 2.05-2.21 (m, 2H); **¹³C NMR** (CDCl₃) δ 163.5 (s), 143.9 (s), 128.6 (s), 128.0 (d), 127.9 (s), 127.2 (s), 86.9 (s), 61.3 (s), 57.2 (s), 53.4 (s), 51.3 (s), 22.9 (s); **MS** (ESI) *m/z* 439.3 (M⁺+H).

To a solution of 3.7 g (6.41 mmol, 1 equiv.) of intermediate **5** in 20 mL of DMF was added sodium hydride (60% in mineral oil, 400 mg, 9.7 mmol, 1.15 equiv.). The reaction mixture was then stirred at rt for 1 h before 3.82 g (10.2 mmol, 1.2 equiv.) oleyl iodide in 10 mL DMF was added. The mixture was stirred additional 4 h before 1 N HCl (100 mL) was added to quench the reaction, and the mixture was extracted with 100 mL of ethyl ether. The combined organic extracts were washed with saturated NaHCO₃ solution, followed by a saturated NaCl solution. Solvents were removed at reduced pressure and the crude mixture was purified on silica column (ethyl acetate-hexanes, 0 to 20%) to give clear oil (Z)-methyl 2-(3-(trityloxy)propylsulfonyl)eicos-11-enoate **6a**. (3.18 g, 4.61 mmol, 72%).

¹H NMR (CDCl₃) δ 7.41-7.51 (m, 6H), 7.20-7.38 (m, 9H), 5.31-5.39 (m, 2H), 3.83 (s, 3H), 3.76-3.81 (m, 1H), 3.15-3.32 (m, 4H), 1.99-2.18 (m, 6H), 1.20-1.40 (m, 26H), 0.90 (t, *J* = 6.8 Hz, 3H); **¹³C NMR** (CDCl₃) δ 167.0 (s), 143.8 (s), 130.0 (s), 129.8 (s), 128.6 (s), 127.9 (d), 127.1 (s), 86.8 (s), 68.7 (s), 61.5 (s), 53.2 (s), 48.6 (s), 31.9 (s), 29.1-29.8 (m), 27.2 (m), 26.2 (s), 22.7 (s), 22.1 (s), 14.2 (s); **MS** (ESI) *m/z* 711.6 (M+Na⁺).

A similar procedure for **6a** was followed, using hexadecyl iodide, to give methyl 2-(3-(trityloxy)propylsulfonyl)octadecanoate **6b** (6.1 g, 9.2 mmol, 88%).

¹H NMR (CDCl₃) δ 7.40-7.51 (m, 6H), 7.21-7.39 (m, 9H), 3.81 (s, 3H), 3.76-3.81 (m, 1H), 3.21-3.46 (m, 4H), 2.14-2.23 (m, 4H), 1.60-1.67 (m, 2H), 1.20-1.40 (m, 26H), 0.88 (t, *J* = 6.8 Hz, 3H); **¹³C NMR** (CDCl₃) δ 167.0 (s), 143.8 (s), 128.6 (s), 127.8 (d), 127.1 (s), 86.8 (s), 68.7 (s), 61.5 (s), 53.2 (s), 48.6 (s), 31.9 (s), 29.1-29.8 (m), 27.2 (m), 26.2 (s), 22.7 (s), 22.1 (s), 14.2 (s); **MS** (ESI) *m/z* 685.3 (M+Na⁺).

To an ice-cooled solution of 800 mg (1.16 mmol, 1 equiv.) of **6a** in 15 mL of THF was added 44 mg (1.16 mmol, 1.0 equiv.) of lithium aluminium hydride. The reaction mixture was then stirred at 0 °C for 10 min. Next, 20 mL water was added dropwise to quench the reaction, and the mixture was extracted twice with 50 mL of ethyl acetate. The combined organic extracts were washed with 1 N HCl, saturated NaHCO₃ solution, and saturated NaCl solution. Solvents were removed at reduced pressure and the crude mixture was purified on silica column (ethyl acetate-hexanes, 5 to 25%) to give the product (Z)-2-(3-(trityloxy)propylsulfonyl)icos-1-en-1-ol **7a** (390 mg, 0.59 mmol, 51%).

Chemical Formula: C₄₂H₆₀O₄S

Exact Mass: 660.4212

7a

¹H NMR (CDCl₃) δ 7.41-7.51 (m, 6H), 7.20-7.38 (m, 9H), 5.31-5.39 (m, 2H), 3.93-4.06 (m, 2H), 3.24 (t, *J* = 6.0 Hz, 2H), 3.11-3.17 (m, 2H), 2.92-3.00 (m, 1H), 2.57 (t, *J* = 6.0 Hz, 2H), 2.16-2.20 (m, 2H), 1.95-2.04 (m, 4H), 1.82-1.92 (m, 1H), 1.61-1.71 (m, 1H), 1.45-1.54 (m, 1H), 1.20-1.40 (m, 22H), 0.88 (t, *J* = 6.8 Hz, 3H); **¹³C NMR** (CDCl₃) δ 143.8 (s), 130.0 (s), 129.8 (s), 128.5 (s), 127.9 (s), 127.1 (s), 86.8 (s), 63.8 (s), 61.5 (s), 59.5 (s), 49.5 (s), 31.9 (s), 29.1-29.8 (m), 27.2 (d), 26.9 (s), 24.3 (s), 22.7 (s), 22.3 (s), 14.1 (s); **MS** (ESI) *m/z* 683.6 (M+Na⁺).

Similarly, **6b** was reduced, worked up, and purified to give (trityloxy)propylsulfonyl)octadecan-1-ol **7b** (2.45 g, 3.86 mmol, 34%).

Chemical Formula: C₄₀H₅₈O₄S

Exact Mass: 634.4056

7b

¹H NMR (CDCl₃) δ 7.40-7.51 (m, 6H), 7.21-7.38 (m, 9H), 3.93-4.06 (m, 2H), 3.24 (t, *J* = 6.0 Hz, 2H), 3.11-3.17 (m, 2H), 2.92-3.00 (m, 2H), 2.57 (t, *J* = 6.0 Hz, 2H), 2.16-2.20 (m, 2H), 1.82-1.92 (m, 1H), 1.61-1.71 (m, 1H), 1.20-1.40 (m, 26H), 0.89 (t, *J* = 6.8 Hz, 3H); **¹³C NMR** (CDCl₃) δ 143.8 (s), 128.5 (s), 127.9 (s), 127.1 (s), 86.8 (s), 63.8 (s), 61.5 (s), 59.5 (s), 49.5 (s), 31.9 (s), 29.1-29.8 (m), 27.2 (d), 26.9 (s), 24.3 (s), 22.7 (s), 22.3 (s), 14.1 (s); **MS** (ESI) *m/z* 657.5 (M+Na⁺).

To a solution of 330 mg (0.6 mmol, 1 equiv.) of **7a** in 5 mL of CH₂Cl₂ was added 0.4 mL of trifluoroacetic acid. Next, the reaction mixture was stirred at rt for 30 min; 30 mL saturated NaHCO₃ solution was added to quench the reaction, and the mixture was extracted with three portions of 50 mL of ethyl acetate. The organic solvents were removed at reduced pressure and the crude mixture was purified on silica column (ethyl acetate-hexanes, 50 to

100%) to give the product (Z)-2-(3-hydroxypropylsulfonyl)icos-11-en-1-ol **8a** (203 mg, 0.49 mmol, 81%).

1H NMR ($CDCl_3$) δ 5.31-5.39 (m, 2H), 3.93-4.06 (m, 2H), 3.81 (t, $J = 6.0$ Hz, 2H), 3.22 (t, $J = 7.2$ Hz, 2H), 2.95-3.03 (m, 1H), 2.07-2.17 (m, 2H), 1.88-2.12 (m, 6H), 1.61-1.71 (m, 1H), 1.45-1.54 (m, 1H), 1.20-1.40 (m, 24H), 0.88 (t, $J = 6.8$ Hz, 3H); **^{13}C NMR** ($CDCl_3$) δ 130.0 (s), 129.8 (s), 64.5 (s), 60.7 (s), 59.6 (s), 49.0 (s), 31.9 (s), 29.1-29.8 (m), 27.2 (d), 26.9 (s), 24.3 (s), 22.7 (s), 14.1 (s); **MS** (ESI) m/z 419.4 ($M^+ + 1$).

Similarly, **7b** was deprotected to give 2-(3-hydroxypropylthio)octadecan-1-ol **8b** (580 mg, 1.61 mmol, 66%).

1H NMR ($CDCl_3$) δ 3.93-4.06 (m, 2H), 3.80 (t, $J = 6.0$ Hz, 2H), 3.21 (t, $J = 7.6$ Hz, 2H), 2.95-3.03 (m, 1H), 2.07-2.17 (m, 2H), 1.88-2.12 (m, 2H), 1.61-1.71 (m, 1H), 1.45-1.54 (m, 1H), 1.20-1.40 (m, 26H), 0.88 (t, $J = 6.8$ Hz, 3H); **^{13}C NMR** ($CDCl_3$) δ 64.5 (s), 60.7 (s), 59.6 (s), 49.0 (s), 31.9 (s), 29.1-29.8 (m), 27.2 (d), 26.9 (s), 24.3 (s), 22.7 (s), 14.1 (s); **MS** (ESI) m/z 361.4 ($M^+ + 1$).

The next protocols involve reaction of both primary alcohols and selective elimination to afford a vinyl sulfone in two steps. To a solution of 3.31 g (7.92 mmol, 1 equiv.) of **8a** and diisopropyl ethylamine (5.1 g, 39.4 mmol, 5 equiv.) in 40 mL of CH_2Cl_2 was added mesyl chloride (2.74 g, 19.0 mmol, 2.4 equiv.). The reaction mixture was then stirred at rt for 2 h. The precipitate was removed by filtration, and the filtrate was washed with saturated $NaHCO_3$ solution and dried over sodium sulfate. The organic solvent was concentrated and the residue was dissolved into acetone (30 mL) followed by NaI (3.6 g, 16 mmol, 3 equiv.) and $NaHCO_3$ (1.0 g, 11.9 mmol, 2.2 equiv.). The reaction mixture was stirred at rt overnight, filtered out and concentrated by evaporation; then, 50 mL water and 50 mL ethyl acetate was added to the residue. The organic layer was collected, the aqueous layer was re-extracted twice with ethyl acetate, and the combined organics were dried over sodium sulfate and then removed at reduced pressure. Next, 100 mL ethyl acetate was added, followed by diisopropylethylamine (5 mL). The reaction mixture was stirred at rt for 3 days. The organic solvent was removed at reduced pressure and the crude mixture was purified on

silica column (ethyl acetate-hexanes, 0 to 15%) to give the product (Z)-2-(3-iodopropylsulfonyl)eicosa-1,11-diene **9a** (3.27 g, 6.42 mmol, 81%).

1H NMR ($CDCl_3$) δ 6.25 (s, 1H), 5.83 (s, 1H), 5.31-5.40 (m, 2H), 3.28 (t, J = 6.4 Hz, 2H), 3.07 (t, J = 7.6 Hz, 2H), 2.41 (t, J = 7.6 Hz, 2H), 2.22-2.30 (m, 2H), 1.94-2.06 (m, 4H), 1.56-1.66 (m, 2H), 1.20-1.40 (m, 22H), 0.88 (t, J = 6.8 Hz, 3H); ^{13}C NMR ($CDCl_3$) δ 146.0 (s), 126.9 (s), 126.6 (s), 122.0 (s), 64.5 (s), 49.7 (s), 28.8 (s), 26.1-26.6 (m), 25.9 (s), 24.6 (s), 24.1 (d), 23.0 (s), 19.6 (s), 11.0 (s); MS (ESI) m/z 533.3 ($M+Na^+$).

A similar two-step procedure was employed using **8b** as the starting material to give 2-(3-iodopropylsulfonyl)octadec-1-ene **9b** (198 mg, 0.41 mmol, 45%).

1H NMR ($CDCl_3$) δ 6.18 (s, 1H), 5.77 (s, 1H), 3.22 (t, J = 6.4 Hz, 2H), 3.02 (m, 2H), 2.34 (t, J = 7.6 Hz, 2H), 2.14-2.23 (m, 2H), 1.50-1.58 (m, 2H), 1.56-1.66 (m, 2H), 1.20-1.40 (m, 24H), 0.81 (t, J = 6.8 Hz, 3H); ^{13}C NMR ($CDCl_3$) δ 145.6 (s), 122.0 (s), 49.5 (s), 28.7 (s), 26.0-26.4 (m), 25.8 (s), 24.4 (s), 22.9 (d), 19.5 (s), 10.9 (s); MS (ESI) m/z 507.3 ($M+Na^+$).

Next, the quaternary ammonium derivatives were introduced. To a solution of 81 mg (0.16 mmol, 1 equiv.) of **9a** in 5 mL ethanol + 1 mL methylene chloride was added trimethylamine in ethanol. Then the reaction mixture was stirred at rt for 4 days. The resulting mixture was concentrated and then dissolved into 0.5 mL ethanol followed by adding 10 mL diethyl ether. The white solid precipitated and the white solid was collected by filtration to give the final product, which was passed through a chloride ion exchange resin to convert to (Z)-3-(eicosa-1,11-dien-2-ylsulfonyl)-*N,N,N*-trimethylpropan-1-aminium chloride **CVS-18** (51 mg, 0.11 mmol, 71%).

¹H NMR (CDCl₃) δ 6.26 (s, 1H), 5.88 (s, 1H), 5.85 (s, 1H), 5.31-5.40 (m, 2H), 3.98-4.40 (m, 2H), 3.59 (s, 3H), 3.45 (s, 9H), 3.18 (t, *J* = 6.8 Hz, 2H), 2.84 (d, *J* = 6.8 Hz, 2H), 2.34-2.43 (m, 4H), 1.57-1.62 (m, 2H), 1.32-1.42 (m, 22H), 0.87 (t, *J* = 6.8 Hz, 3H); **¹³C NMR** (CDCl₃) δ 149.1 (s), 130.0 (s), 129.7 (s), 125.6 (s), 64.5 (s), 53.7 (s), 52.0 (s), 48.6 (s), 44.9 (s), 31.9 (s), 29.0-29.7 (m), 27.7 (s), 27.2 (d), 22.6 (d), 16.5 (s), 14.1 (s); **MS** (ESI) *m/z* 442.5 (M⁺).

A similar procedure for **CVS-18** was used to give *N,N,N*-trimethyl-3-(octadec-1-en-2-ylsulfonyl)propan-1-aminium chloride **CVS-16** (21 mg, 0.05 mmol, 85%).

¹H NMR (CD₃OD) δ 6.25 (s, 1H), 5.98 (s, 1H), 3.48-4.55 (m, 2H), 3.26 (s, 2H), 3.16-3.22 (m, 11H), 2.45 (t, *J* = 7.6 Hz, 2H), 2.18-2.28 (m, 2H), 1.60-1.67 (m, 2H), 1.32-1.42 (m, 24H), 0.90 (t, *J* = 6.8 Hz, 3H); **¹³C NMR** (CD₃OD) δ 149.2 (s), 125.1 (s), 64.3 (s), 52.2 (m), 48.4 (s), 31.7 (s), 29.0-29.4 (m), 28.8 (s), 27.6 (s), 22.3 (d), 16.2 (s), 13.0 (s); **MS** (ESI) *m/z* 416.5 (M⁺).

Modified protocols were required to prepare the two shorter chain analogs. Thus, bromo ester **12** (12.06 g, 36.0 mmol) was dissolved in acetone (200 mL), K₂CO₃ (7.45 g, 54.0 mmol, 1.5 equiv.) was added, followed by methyl 2-mercaptoacetate **11** (4.2 g, 39.5 mmol, 1.1 equiv.) and heated at reflux for 2 h. After cooling to room temperature the mixture was filtered and the white solid was washed twice with 25 mL acetone. Combined organic solvents were evaporated under reduced pressure, and the residue was purified on silica column (ethyl acetate-hexanes, 15 to 40%) to yield the pure product ethyl 2-(3-methoxy-3-oxopropylthio)tetradecanoate **13a** (12.0 g, 32.0 mmol, 89%).

¹H NMR (CDCl₃) δ 4.14-4.25 (m, 2H), 3.69 (s, 3H), 3.20-3.26 (m, 1H), 2.81-2.92 (m, 2H), 2.56-2.64 (m, 2H), 1.81-1.92 (m, 2H), 1.60-1.68 (m, 2H), 1.21-1.48 (m, 21H), 0.87 (t, *J* = 6.8 Hz, 3H); **¹³C NMR** (CDCl₃) δ 172.7 (s), 172.1 (s), 61.1 (s), 51.7 (s), 46.8 (s), 34.4 (s), 31.9 (s), 31.4 (s), 29.2-29.6 (m), 27.3 (s), 26.2 (s), 22.7 (s), 14.2 (s), 14.1 (s); **MS** (ESI) *m/z* 375.4 (M⁺+1).

As for the two long chain analogs, the bis primary alcohols were prepared, in this case by reducing both esters simultaneously. Thus, to an ice-cooled solution of 3.8 g (13.1 mmol, 1 equiv.) of **13a** in 100 mL of THF was added 2.0 g (52.6 mmol, 4.0 equiv.) of lithium aluminum hydride. Then the reaction mixture was stirred at 0 °C for 10 min. Then the reaction temperature was raised to 60 °C overnight, then cooled down to rt. The resulting mixture was filtered, and concentrated before 100 mL ethyl acetate was added. The organic layer was washed with 1 N HCl, saturated NaHCO₃ solution, followed by a saturated NaCl solution. Solvent was removed at reduced pressure and the crude mixture was purified on silica column (ethyl acetate-hexanes, 25 to 50%) to give the product 2-(3-hydroxypropylthio)tetradecan-1-ol **14a** (2.6 g, 11.8 mmol, 90%).

A similar procedure to reduce **13b** to give 2-(3-hydroxypropylthio)octan-1-ol **14b** (1.87 g, 8.51 mmol, 88%).

1H NMR ($CDCl_3$) δ 3.76 (t, J = 6.0 Hz, 2H), 3.46-3.52 (m, 1H), 2.58-2.76 (m, 3H), 2.03 (s, 2H), 1.81-1.89 (m, 2H), 1.45-1.62 (m, 3H), 1.21-1.44 (m, 8H), 0.88 (t, J = 6.8 Hz, 3H); **^{13}C NMR** ($CDCl_3$) δ 63.9 (s), 61.5 (s), 49.6 (s), 32.4 (s), 31.8 (s), 31.7 (s), 29.1 (s), 27.1 (s), 26.7 (s), 22.6 (s), 14.0 (s); **MS** (ESI) m/z 203.3 (M^+-OH).

The corresponding sulfide **14a** (10.6 g, 34.8 mmol, 1 equiv.) was dissolved in CH_2Cl_2 (180 ml) and *m*-CPBA (15.0 g, 87.0 mmol, 2.5 equiv.) was added to it at 0 °C (ice bath). The reaction mixture was allowed warm to rt and stirred for 8 h at rt. Volatiles were evaporated under reduced pressure. The residue was taken up in ethyl acetate (150 mL) and washed with 10% Na_2SO_3 , saturated $NaHCO_3$, and brine. The organic layer was then dried over anhydrous Na_2SO_4 . The volatiles were evaporated under reduced pressure to yield the crude mixture that was purified on silica column (ethyl acetate-hexanes, 30 to 80%) to yield the pure product 2-(3-hydroxypropylsulfonyl)tetradecan-1-ol **15a**. (9.0 g, 26.8 mmol, 77%)

1H NMR ($CDCl_3$) δ 3.90-4.05 (m, 2H), 3.79 (t, J = 6.0 Hz, 2H), 3.22 (t, J = 7.6 Hz, 2H), 2.94-3.02 (m, 1H), 2.49 (s, 2H), 2.06-2.15 (m, 2H), 1.84-1.95 (m, 1H), 1.52-1.72 (m, 1H), 1.45-1.55 (m, 1H), 1.22-1.40 (m, 19H), 0.88 (t, J = 6.8 Hz, 3H); **^{13}C NMR** ($CDCl_3$) δ 64.6 (s), 60.6 (s), 59.6 (s), 49.4 (s), 31.4 (s), 29.3-29.6 (m), 26.9 (s), 24.3 (s), 24.2 (s), 22.5 (s), 14.0 (s); **MS** (ESI) m/z 337.3 (M^++H).

Sulfide **14b** was oxidized as described above for sulfide **14a** to give methyl 2-(3-hydroxypropylsulfonyl)octan-1-ol **15b** (1.79 g, 7.1 mmol, 81%).

1H NMR ($CDCl_3$) δ 3.90-4.05 (m, 2H), 3.75 (t, J = 6.0 Hz, 2H), 3.21 (t, J = 7.6 Hz, 2H), 2.94-3.02 (m, 1H), 2.82 (s, 2H), 2.03-2.11 (m, 2H), 1.84-1.95 (m, 1H), 1.58-1.69 (m, 1H), 1.42-1.52 (m, 1H), 1.22-1.40 (m, 7H), 0.86 (t, J = 6.8 Hz, 3H); **^{13}C NMR** ($CDCl_3$) δ 64.6

(s), 60.6 (s), 59.6 (s), 49.4 (s), 31.4 (s), 29.0 (s), 26.9 (s), 24.3 (s), 24.2 (s), 22.5 (s), 14.0 (s); **MS** (ESI) m/z 253.3 ($M^+ + H$).

As above, the vinyl sulfones were formed and primary iodide introduced in a two-step protocol. Thus, to a solution of 2.0 g (7.92 mmol, 1 equiv.) of **15b** and diisopropyl ethylamine (5.1 g, 39.4 mmol, 5 equiv.) in 40 mL of CH_2Cl_2 was added mesyl chloride (2.74 g, 19.0 mmol, 2.4 equiv.). Then the reaction mixture was stirred at rt for 2 h. The mixture was filtered, and the filtrate was washed with saturated $NaHCO_3$ solution and then dried over sodium sulfate. The organic solvent was concentrated. The residue was dissolved into acetone (30 mL) followed by NaI (3.6 g, 16 mmol, 3 equiv.) and $NaHCO_3$ (1.0 g, 11.9 mmol, 2.2 equiv.). The reaction mixture was stirred at rt overnight, filtered, and concentrated. Then, 50 mL water and 50 mL ethyl acetate were added to the residue, and the organic layer was collected and the aqueous layer re-extracted twice with ethyl acetate. The combined organics were dried over sodium sulfate and then removed at reduced pressure. Next, 100 mL ethyl acetate was added followed by diisopropylethylamine (5 mL). The reaction mixture was stirred at room temperature for 3 days. The organic solvent was removed at reduced pressure and the crude mixture was purified on silica column (ethyl acetate-hexanes, 0 to 15%) to give the product 2-(3-iodopropylsulfonyl)oct-1-ene **16b** (2.23 g, 6.49 mmol, 82%).

Chemical Formula: $C_{11}H_{21}IO_2S$
Exact Mass: 344.0307
16b

1H NMR ($CDCl_3$) δ 6.25 (s, 1H), 5.83 (s, 1H), 3.28 (t, $J = 6.8$ Hz, 2H), 3.08 (t, $J = 7.6$ Hz, 2H), 2.41 (t, $J = 7.6$ Hz, 2H), 2.21-2.30 (m, 2H), 1.52-1.63 (m, 2H), 1.25-1.42 (m, 6H), 0.89 (t, $J = 6.8$ Hz, 3H); **^{13}C NMR** ($CDCl_3$) δ 145.9 (s), 122.0 (s), 49.7 (s), 28.3 (s), 26.6 (s), 25.6 (s), 24.6 (s), 23.1 (s), 19.4 (s), 10.9 (s); **MS** (ESI) m/z 367.2 ($M + Na^+$)

A similar procedure to convert **15a** to 2-(3-iodopropylsulfonyl)tetradec-1-ene **16a** (346 mg, 0.81 mmol, 69%).

Chemical Formula: $C_{17}H_{33}IO_2S$
Exact Mass: 428.1246
16a

1H NMR ($CDCl_3$) δ 6.23 (s, 1H), 5.81 (s, 1H), 3.28 (t, $J = 6.4$ Hz, 2H), 3.07 (t, $J = 7.6$ Hz, 2H), 2.40 (t, $J = 7.6$ Hz, 2H), 2.21-2.30 (m, 2H), 1.52-1.63 (m, 2H), 1.25-1.42 (m, 18H), 0.87 (t, $J = 6.8$ Hz, 3H); **^{13}C NMR** ($CDCl_3$) δ 145.9 (s), 122.0 (s), 49.7 (s), 28.7 (s), 26.1-26.6 (m), 25.9 (s), 24.6 (s), 23.0 (s), 19.5 (s), 11.0 (s); **MS** (ESI) m/z 451.3 ($M + Na^+$).

A procedure similar to that described above for **CVS-18** was used to obtain *N,N,N*-trimethyl-3-(tetradec-1-en-2-ylsulfonyl)propan-1-aminium chloride **CVS-12** (**17a**, 23 mg, 0.058 mmol, 58%).

1H NMR (CD_3OD) δ 6.25 (s, 1H), 5.98 (s, 1H), 3.48-3.56 (m, 2H), 3.27 (s, 2H), 3.16-3.22 (m, 11H), 2.45 (t, $J = 7.6$ Hz, 2H), 2.20-2.28 (m, 2H), 1.60-1.66 (m, 2H), 1.26-1.44 (m, 16H), 0.90 (t, $J = 6.8$ Hz, 3H); **^{13}C NMR** (CD_3OD) δ 149.2 (s), 125.1 (s), 64.3 (s), 52.2 (m), 31.4 (s), 28.8-29.3 (m), 27.7 (s), 26.7 (s), 22.3 (s), 16.2 (s), 13.0 (s); **MS** (ESI) m/z 360.3 (M^+).

A procedure similar to that described above for **CVS-18** was used to provide *N,N,N*-trimethyl-3-(oct-1-en-2-ylsulfonyl)propan-1-aminium chloride **CVS-6** (**17b**, 11 mg, 0.035 mmol, 72%).

1H NMR ($CDCl_3$) δ 6.28 (s, 1H), 5.88 (s, 1H), 4.05-4.13 (m, 2H), 3.43 (s, 9H), 3.22 (t, $J = 6.8$ Hz, 2H), 2.38-2.46 (m, 4H), 1.55-1.65 (m, 2H), 1.28-1.42 (m, 6H), 0.89 (t, $J = 6.8$ Hz, 3H); **^{13}C NMR** ($CDCl_3$) δ 149.1 (s), 125.8 (s), 54.1 (s), 48.3 (s), 31.5 (s), 29.6 (s), 28.6 (s), 27.7 (s), 22.5 (s), 16.6 (s), 14.0 (s); **MS** (ESI) m/z 276.4 (M^+).

ATX assay

ATX inhibition was performed using an ATX Inhibitor Screening Kit (K-4200, Echelon Biosciences, Inc. Salt Lake City, UT). Stock solutions (1 mM) of each compound were made in DMSO and then diluted with water to the appropriate experimental concentration. Fourteen final inhibitor concentrations from 0.1 to 10,000 nM were employed to calculate K_i values. DMSO was spiked into each reaction mixture to equalize vehicle concentrations. The assay employs the fluorescence-quenched, ATX analogue FS-3 as the ATX substrate²⁵ and recombinant, human ATX purified from Sf9 insect cells as the enzyme source. Compounds were pre-incubated with the enzyme at 25° C for 10 minutes, after which FS-3 was added. The rate of fluorescence increase was measured between 5 and 25 minutes after substrate addition. In all circumstances fluorescence increase was linear during this time window. Rates were normalized to control reactions that contained all reaction components except test compound.

Dilution/dialysis for reaction of 10b (CVS-16) or 21 (PVS-16) with ATX.

ATX was incubated with compound **10b** or **21** (0, 1 or 10 μ M) for two minutes in 50 mM Tris-Cl pH 8.0, 5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 140 mM NaCl, 1 mg/ml Fatty Acid Free BSA. Half of each reaction was placed in a centrifugal filtration device (Millipore) with a molecular weight cutoff of 30 kDa and subjected to repeated rounds of concentration and dilution using buffer absent of test compound. ATX activity was then assessed in each sample by monitoring FS-3 (1 μ M) fluorescence increase over time and compared to the activity of ATX not incubated with test compound or subjected to dialysis.

Cell culture

Cell lines were acquired from the American Type Culture Collection or ATCC (Manassas, VA). Human cancer cell lines SKOV-3, OVCAR-3, PC-3 and MDA-MB-231 cells were maintained in RPMI (Mediatech Inc., Manassas, VA) supplemented with 10% fetal bovine serum or FBS (Sigma, St. Louis, MO). MeWo fibroblast malignant melanoma cells were cultured similarly, but supplemented with only 5% FBS. HT-29 human colon cancer cells and SB-2 human non-metastatic melanoma cells were maintained in DMEM (Mediatech) supplemented with 10% fetal bovine serum.

Cell viability assay

Approximately 5,000 cells were grown in each well of a 96-well plate for 24 h prior to the addition of CVS-16 (10b) compound at the indicated concentrations for 48 h. The assay was then performed by removing medium from the 96-well plate and replacing it with serum free media containing CellTiter-Blue® reagent (Promega Corporation, Madison, WI) and incubating for 4-6 h at 37 °C. Then the fluorescent absorbance was measured with a microplate reader, the SpectraMax M2 model (Molecular Devices, Sunnyvale, CA).

Wound healing assay

Cells were seeded in triplicates in 24-well plates and then grown to confluence. 'Wounds' were then created using a pipette tip. The wells were then repeatedly rinsed using serum-free medium followed by the addition of 18:1 LPA (Avanti Polar Lipids, Alabaster, AL) or CVS-16 (10b) to the wells and incubated for 24 h. Photomicrographs were taken of the wells, at least six per well, and treatments were performed in triplicate. The distance between confluent monolayers was measured.

Animal model of melanoma

Six-week old female athymic nude mice acclimated to the animal facility for one week prior to the study commencement. Animals were anesthetized before tumor cell injection into their right flank with Glycosan Extracel® (BioTime, Inc, Alameda, CA) containing approximately 1×10^6 MeWo cells per 0.15 mL injection. Extracel® was used in this study over traditional Matrigel to eliminate potential interference of exogenous mouse growth factors which could have affected our *in vivo* study. In addition, Glycosan Extracel® allows us to conserve resources through the achievement of a 100% tumor efficiency rate, whereby this rate is typically unachievable otherwise. Injected mice were measured for tumor formation, tumor volume, body weight and body conditioning scores. After three weeks,

100% of mice displayed tumor formation at which time they were randomized into treatment groups; PBS control (n=10), CVS-16 (10b) at 20 mg/kg (n=5) or 50 mg/kg (n=5), DMF control (n=10), HA-130 at 30 mg/kg (n=5) and PF-8380 at 30 mg/kg (n=5) dose per 0.1 mL injection. Mice in each treatment group were anesthetized (2-4% isofluorane) before i.p. treatment injections three times a week over the course of the 65-day study. The animals were euthanized according to the animal use protocol approved by the University of Georgia IACUC committee. The tumor volume (mm³) was calculated using the equation: tumor volume = (width)² × length/2, and then graphed using GraphPad Prism (La Jolla, CA). The tumor volume for each group and overall significance was plotted.

Tumor specimen analysis

Melanoma tumors were dissected from the right flank of athymic nude mice and flash-frozen with cryomatrix (Thermo Fisher Scientific Inc, Waltham, MA) in 2-methylbutane (Sigma) and cooled to -140°C. Cryopreserved tumors were cut in 9 µm sections using a cryostat (Thermo Fisher Scientific) and then mounted on microscope slides. Hematoxylin and eosin staining was performed according to standard protocols and processed for pathological evaluation.

For tissues stained with antibodies specific for immunofluorescence, tissue sections first were blocked for 30 min in 5% donkey serum (Jackson ImmunoResearch Laboratories Inc, West Grove, PA) diluted in phosphate-buffered saline (PBS) for 30 min at room temperature. The tumor sections were then incubated in primary antibodies at 4°C overnight using a humidity chamber. The following day slides were washed with PBS and the primary antibody was detected using a fluorescent secondary antibody. After washing the slides again with PBS, they were mounted with Permount (Thermo Fisher Scientific) and imaged using an X71 inverted microscope (Olympus, Center Valley, PA) at 20x magnification. Overlapping pictures were aligned to generate an image of an entire tumor cryosection. The tumor area was automatically calculated using the polygon tool in Image-Pro Precision software (Media Cybernetics, Inc., Rockville, MD), then the Ki-67 pixel intensity was automatically determined. The average pixel staining intensity per area measurement was plotted.

Serum analysis

Whole blood was collected from animals at necropsy and placed into a Becton Dickinson Microtainer® tube with serum separator additive and allowed to clot for at least 20 min in a vertical position at room temperature. The tubes were then inverted five times prior to centrifugation at 2000 RCF for 10 min at room temperature. The serum was transferred to a glass vial and stored in -80°C until use. Approximately 200 µl of serum was obtained from each mouse. From that, 100 µl of serum was analyzed for cytokines, chemokines, growth factors and interleukins using the mouse Bio-Plex, 23-Plex panel (Bio-Rad, Hercules, CA) and following the instructions provided by the manufacturer. The assay plates were measured using the Bio-Plex Multiplex Suspension Array system (Bio-Rad). Serum samples were measured via comparison to an 8-point standard dilution series included as an integral component of the high-throughput assay. For analysis of ATX, the remaining 100 µl of serum was used processed using the mouse ectonucleotide pyrophosphatase/

phosphodiesterase family member 2 (ENPP2) ELISA kit according to the instructions provided by the manufacturer (MyBioSource.com, San Diego, CA).

Statistics

The statistical differences were analyzed using an analysis of variance (ANOVA) test, followed by Bonferroni's multiple comparison test between groups using GraphPad Prism. When comparing only two groups, the Student's *t*-test was used. Where it is indicated in the figures, **p* < 0.05 ***p* < 0.01 and ****p* < 0.001 indicate the levels of significance.

RESULTS

Chemical synthesis of vinyl sulfone analogs

Although palladium or copper-promoted reaction of sulfinic acids with alkyl halides or triflates can provide vinyl sulfones in good yield²⁶, the harsh conditions (80 °C) were not appropriate for these analogs. Our strategy for the synthesis of desired targets involves conversion of the key intermediate β -hydroxy sulfones **8** to vinyl sulfones **9**. In addition, the quaternary ammonium salts **10** and **17** were prepared in the final step to avoid difficulties in purification of reactive electrophilic species.

Two different synthetic routes were carried out, based on the availability of the starting materials. The vinyl sulfone analogues with the 16:0 and 18:1 alkyl chains were prepared as shown in **Figure 1**. We selected these chain lengths based on the known bioactivity of LPA agonists for the LPARs, with the expectation that these would be the best probes for the ATX active site. The formation of the thioether **3** could be performed by displacement of the bromide anion **1** with bromide in good yield (98%). The primary hydroxyl group was then tritylated (TrCl, Et₃N) to give compound **4**. Conversion of sulfide **4** to sulfone **5** was accomplished by oxidation with *m*-CPBA in dichloromethane. In order to alkylate compound **5** regioselectively, we chose sodium hydride as the base so that the reaction could be performed at room temperature in moderate yield; the regioisomer was not detected.

Next, the reduction of ester and removal of the trityl group was carried out to give the diol **8**. However, preliminary attempts to reduce the ester were unsuccessful. An unexpected over-reduction of hydroxyl to methyl was observed using either excess lithium aluminum hydride or sodium borohydride. Possible reasons for the over-reduction include excess reductant, long reaction time, reaction temperature, or the proximity of the β -sulfone moiety, but this was not further explored. After optimization, moderate yields of reduction (34-51%) could be obtained by using 1 equiv. of lithium aluminum hydride in THF at 0 °C for 10 min. The conversion of diols **8** to vinyl sulfone **9** involved a three-step sequence, although no intermediates need to be isolated. Conversion of **8** to the corresponding mesylate and subsequent iodination afforded the iodo intermediates. Next, the β -iodo sulfones were β -dehydroiodinated by treatment with diisopropylethylamine (DIPEA) in EtOAc to give the desired vinyl sulfones. Importantly, γ -iodoalkyl groups were stable in the presence of DIPEA, which allowed us to install the quaternary ammonium derivative in a subsequent step. Thus, the corresponding quaternary ammonium salts could be obtained by treating the remaining iodoalkyl group with trimethylamine in a mixture of ethanol and dichloromethane

for 4 days. Recrystallization of crude salts from CH₃OH-Et₂O provided the final targets **10** (CVS-18 and CVS-16) as white solids. (**Figure 1**)

In an effort to synthesize the shorter chain vinyl sulfone, which we hypothesized would be more water-soluble and still retain ATX inhibitory activity, we took the advantage of commercially available α -bromo esters **12**, which reacted with thiol directly to give corresponding sulfide (**Figure 2**). Excess lithium aluminum hydride was employed to reduce ester groups to diols **14**. Oxidation with *m*-CPBA in dichloromethane at 0 °C converted the sulfides **14a,b** to sulfones **15a,b** in good yield. Then final targets **17a** and **17b** (CVS-6 and CVS-12) were prepared from sulfones **15a, b** in an analogous fashion described previously for the structurally-similar analogue **10a**. (**Figure 2**)

A non-choline-like head vinyl sulfone **21** (PVS-16) that retained the 16:0 alkyl chain was synthesized as a negative control. Alkylation of commercially available compound **18** by using sodium hydride (60 % in mineral oil) as base proceeded in high yield (95 %). After the hydrophobic chain was installed, phenyl vinyl sulfone compound **21** could be achieved by mesylation/iodination and elimination as employed above. (**Figure 3**)

Kinetic inhibition of ATX

The analysis of the kinetic inhibition of ATX demonstrated that the enzymatic activity decreased in a time-dependent manner. However, the time course of inhibition is very rapid since pre-incubating ATX with the compound CVS-16 (**10b**) for just 2 minutes results in complete inhibition. Different hydrophobic chain lengths were synthesized to study the structure-activity relationship of the compound. The inhibition of ATX by the vinyl sulfone ATX analogues CVS-18 (**10a**) and CVS-16 (**10b**) was tested using an ATX inhibitor screening kit (Echelon Biosciences, Inc.) in a dose-response mode (Table 1). ATX activity was measured by the hydrolysis of the fluorogenic ATX analogue FS-3, which has a K_M value of 6.3 μ M²⁵. The results showed that all analogues except the shorter-chain analogue CVS-6 (**17b**) inhibited ATX effectively, with C18:1 ATX showing the greatest inhibition²⁷. Non-c quaternary ammonium head group vinyl sulfone PVS-16 (**21**) showed no binding effect at the highest test concentration, which reveals the importance of quaternary ammonium head group for achieving the binding affinity. (**Table 1**)

We tested the irreversible nature of compound CVS-16 (**10b**) by performing a washout experiment (**Supplementary Figure 1**). ATX was pre-incubated with compound CVS-16 (**10b**) or buffer for two minutes, after which time half of each reaction mixture was placed in a centrifugal filtration device. Over approximately one hour, samples were subjected to multiple rounds of concentration and dilution, resulting in over 1000-fold dilution of compounds CVS-16 (**10b**) and PVS-16 (**21**). ATX activity was then assessed by monitoring FS-3 hydrolysis. Attempted wash out of CVS-16 (**10b**) did not decrease the ATX inhibition, a finding consistent with CVS-16 (**10b**) irreversibly binding to the enzyme. PVS-16 showed no evidence of inhibiting ATX activity whether or not the compound was subjected to the washout experiment.

***In vitro* biological activity**

In order to determine whether CVS-16 (**10b**) possessed *in vitro* biological activity, studies were conducted to explore the functional potency of CVS-16 (**10b**) against cell viability. For these studies, we used multiple cancer cell lines to represent a wide range of subtypes including HT-29 (colon), PC-3 (prostate), MDA-MB-231 (breast), MeWo (melanoma), SB-2 (melanoma), OVCAR-3 (ovarian) and SKOV-3 (ovarian). In all cell lines, the CVS-16 (**10b**) compound reduced cell viability to some extent (**Figure 4A**) with significant activity of CVS-16 (**10b**) observed between 0.5-5 μ M (**Figure 4B**). The IC₅₀ values of CVS-16 (**10b**) are listed in **Table 2** and the IC₅₀ values of PVS-16 are in **Supplementary Figure 2**). Interestingly, CVS-16 (**10b**) had strong activity against MDA-MB-231 cells (IC₅₀ = 0.39 μ M) in comparison to the other cell types, but it was effective against all cell lines tested (*p > 0.05). As a control, we also examined the effect of PVS-16 (**21**) cell against viability and observed that most cell types were unaffected by its presence (**Figure 4C**), especially at relevant concentrations (**Figure 4D**).

Next we assessed confluent monolayer wounding and measured subsequent closure using MDA-MB-231, OVCAR-3, SKOV-3 and MeWo cells in the presence or absence of LPA (18:1, 1 μ M) and/or CVS-16 (**10b**, 5 μ M). For all cell lines examined, we observed that CVS-16 (**10b**) significantly (*p<0.05 vs. untreated) inhibited wound healing, which encompasses cell proliferation and migration from a denuded front that eventually closes the gap between confluent cell monolayers on either side (**Supplementary Figure 3**). In addition, the CVS-16 (**10b**) analog (5 μ M) displays a significant reduction (over 75%) in both OVCAR-3 and MDA-MB-231 cell lines. Unexpectedly in most cases, LPA was not able to overcome the inhibition of CVS-16 (**10b**) on wound closure when these two reagents were combined.

Since the *in vitro* data thus far suggested that CVS-16 (**10b**) has a significant impact against MeWo cells, we wanted to compare the functional activity of this compound against other state-of-the-art ATX inhibitors; for this comparison, we selected: HA-130 ^{15a} and PF-8380 ^{15b}. Since melanoma cells are notoriously and intrinsically resistant to traditional chemotherapy, we also included several negative controls: dacarbazine (DTIC), paclitaxel (PTX/TAX), vincristine (VCR) and cisplatin (CDDP). Indeed, the chemotherapy had little impact on MeWo cells – never dropping below 50% cell viability (**Figure 5**). In contrast, the ATX inhibitors CVS-16 (**10b**) and HA-130 were able to reduce cell viability below 25%. Although there was no significant difference in activity between HA-130 with either of the other ATX inhibitors, CVS-16 (**10b**) was significantly different from PF-8380 (*p<0.05) and chemotherapy (**p<0.01).

Inhibition of melanoma progression and angiogenesis

In light of the fact that the data thus far suggested CVS-16 (**10b**) had significant biological activity *in vitro*, we wanted to next assess whether it also had *in vivo* activity. Since there is a dearth of clinical therapeutics to treat melanomas lacking BRAF mutations, we examined CVS-16 (**10b**) in an animal model of melanoma. After establishing small pigmented melanoma tumors using MeWo cells injected with Glycosan Extracel®, the mice were randomized into groups and then treated every-other-day with either diluent (control),

CVS-16 (**10b**) at 20 mg/kg or CVS-16 (**10b**) at 50 mg/kg, which began 21 days post tumor cell injection. Prior to day 45, the tumor sizes in all groups appeared identical, but then began to diverge with the control groups displaying a linear rate of growth (**Figure 6A**). The group of mice treated with 20 mg/kg of CVS-16 (**10b**) also showed linear growth around day 50. On day 57, the tumor sizes between groups achieved statistical significance (**Figure 6B**, * $p < 0.05$, 50-40 mg/kg CVS-16 (**10b**) vs. control; day 65 -*** $p < 0.001$, 50-40 mg/kg CVS-16 (**10b**) vs. control) and this trend continued through the conclusion of the study, whereby control groups reached maximum allowable tumor volume. Mouse weight data was also collected (**Supplementary figure 4**).

Of note is that on the 46th day of the study, the group of mice treated with 50 mg/kg of CVS-16 (**10b**) looked severely dehydrated and required medical intervention. Unfortunately, one mouse in the 50 mg/kg group (with a very small tumor and no signs of ascites upon necropsy) rapidly declined in health and then died the following day, even after veterinarians helped treat the mouse for this condition. Thus, on the 48th day, we reduced the concentration of CVS-16 (**10b**) to 40 mg/kg (marked with an arrow) so that the experiment could continue without additional mice succumbing to a possible unspecified side effects of CVS-16 (**10b**) at 50 mg/kg. Subsequently, all of the mice in this group were then fed a special diet to curb dehydration, in addition to their regular chew pellets and no others died before the date of necropsy.

Upon necropsy, we collected tissues and serum from all mice remaining in the study. We then measured the mouse serum for 25 different secreted factors, including cytokines, chemokines, interleukins, etc. Understanding that many interleukins would only be expressed by animals with immune-intact systems, we also coupled this study to another whereby we treated C57/Bl6 animals ($n=20$) with CVS-16 (**10b**) and measured their serum (data not shown). Interestingly, the most significant decrease in chemokines among treated animal serum from the present study shown herein was the keratinocyte chemoattractant (KC, also referred to as Chemokine C-X-C Motif Ligand 1 (CXCL1) or the Melanoma Growth Stimulating Activity Alpha protein) (**Figure 6C**, * $p < 0.05$, *** $p < 0.001$). The KC/CXCL-1 chemokine is homologous to the human growth regulated oncogene alpha (GRO-alpha), which is regulated by ATX²⁸ and associated with cancer progression²⁹. Since chemokines are often significantly upregulated during tumorigenesis and melanoma tumor cells secrete KC/CXCL-1³⁰, which exerts signaling effects on endothelial cells, the significant reduction of KC/CXCL-1 suggests inhibition of tumor progression.

We then sectioned and stained tumor specimens with hematoxylin and eosin for pathology analysis of the tissues. Intriguingly, the percent of necrotic tissue within the tumor of the animals treated at the highest concentration of CVS-16 (**10b**) (40-50 mg/kg) had significantly more necrosis than control (** $p < 0.01$) and 20 mg/kg treated animals (* $p < 0.05$) (**Figure 6D**). In addition, the endothelial cells and state of the tumor specimens was examined. One hundred percent of the control specimens contained viable endothelial cells. This was in contrast to 20 mg/kg treated animals, of which 50% contained only viable endothelial cells present and the other 50% of specimens displayed mixed areas with viable endothelial cells and also necrotic endothelial cells (**Figure 6E**). Most interesting was that 100% of specimens from animals treated with 40-50 mg/kg of CVS-16 (**10b**) contained

necrotic endothelial cells in close proximity to necrotic malignant cells. Taken together, the data suggests an anti-angiogenesis mechanism of action for CVS-16 (**10b**). In support of this idea, we stained tumor sections with Ki-67 and detected no significant differences between specimens (data not shown).

In order to understand whether CVS-16 (**10b**) had biological activity *in vivo*, we isolated the mouse serum at necropsy for analysis of circulating ATX. We detected a significant reduction in the ATX of treated animals, compared to control (**Figure 6F**, * $p < 0.05$ vs. control). This suggests that CVS-16 (**10b**)-treated tumors manifested significant reduction in the expression and production of ATX. Taken together with the previous data, this indicates that the vinyl sulfone inhibits ATX which affects angiogenesis and melanoma progression *in vivo*.

Using the same *in vivo* model of melanoma progression, we also tested the efficacy of HA-130 and PF-8380. Surprisingly, neither of these ATX inhibitors reached statistical significance against the continuous growth of tumors from MeWo cell inoculation in mice (**Figure 7A and 7B**). Upon necropsy, we collected the serum to analyze circulating ATX levels and observed no significant difference between the treated animals and the control group (**Figure 7C**). This corroborated the results we observed with solid tumor growth, suggesting the compounds were not able to have a significant impact on ATX. Taken together, this further suggests that the vinyl sulfone CVS-16 (**10b**) has superior functional activity in comparison to these other ATX inhibitors.

DISCUSSION

Herein we report the synthesis and biological activity of a vinyl sulfone analog of LPC, which acts as an active-site targeted irreversible inhibitor of ATX *in vitro*, and also shows activity against melanoma *in vitro* and *in vivo*. In addition to enzymatic inhibition of ATX by the vinyl sulfone analogs *in vitro*, we observed the reduction of cell viability and migration among multiple cancer cell types *in vitro*. Most importantly, we observed the inhibition of tumor progression using an *in vivo* model of melanoma and measured a reduction of ATX in the serum of treated animals. Taken together, our data suggests that the vinyl sulfone CVS-16 (**10b**) is a potent small molecule inhibitor of ATX with biological activity against its target, which reduces viability of cancer cells and inhibits angiogenesis necessary for tumor progression.

Our data also suggests that the vinyl sulfone CVS-16 (**10b**) compares very favorably against other compounds synthesized to inhibit ATX, like HA-130 and PF-8380. The first known inhibitor of ATX was L-histidine and it was limited in application due to the millimolar concentrations of L-histidine required to inhibit the lysophospholipase D activity of ATX^{15g}. Later work identified α -halophosphonate analogs of LPA as potent ATX inhibitors³¹, as well as aromatic phosphonates^{15i, 32}. This report was the first to provide a proof-of-concept study that led to further innovation, as reviewed by Parrill, Baker and coworkers³³. For example, using a chemical library screening approach and ~40,000 drug-like small molecules, thiazolidinediones were identified as ATX inhibitors that could be enhanced by the addition of a boronic acid moiety to achieve lower IC₅₀ levels^{15a}. Another

screen of a small-molecule library yielded several ATX inhibitors, which lead to the synthesis of analogues based on their structural motifs and further validated ATX as a target for melanoma ³⁴.

We, and other groups, have also taken advantage of cyclic phosphatidic acid, a naturally occurring molecule that inhibits ATX and is an analogue of ATX. Manipulating the compound structure to yield 3-carba analogues of cyclic phosphatidic acid produced potent inhibitors of ATX that were effective *in vivo* ^{8a}. Similarly, a phosphonothionate analogue of carba cyclic phosphatidic acid also yielded a compound that inhibited the lysophospholipase D activity of ATX, the viability of melanoma cells and reduced melanoma metastasis *in vivo* ^{8b}. A recent study reported the evaluation of the stereoisomers of 3-carba cyclic phosphatidic acid, which are agonists of the LPA5 receptor, yet inhibits melanoma metastasis *in vivo* ³⁵.

There are several differences between these reports and the current inhibitor. First and foremost among these differences is the use of a vinyl sulfone moiety as a mild electrophile that is targeted to the active site by an alkyl chain and a quaternary ammonium derivative that recapitulate key recognition elements of the LPC substrate structure. As a result, CVS-16 (**10b**) is an irreversible, “suicide” inhibitor of ATX. There is only one other reported irreversible inhibitor of ATX, a series of monofluoro- and difluoromethyl phenyl alkyl phosphodiester that liberate a reactive quinone methide upon hydrolysis by ATX ³⁶. This irreversible inhibitor lacks the quaternary ammonium derivative for targeting, which we show is crucial for activity of the LPC-mimicking vinyl sulfones; lacking the quaternary ammonium derivative, analog PVS-16 (**21**) is essentially inactive. In addition, the reactive quinone methide is released as a diffusible highly reactive electrophile, rather than the more selective Thr-targeted vinyl sulfone.

Secondly, in previous studies we focused exclusively on advanced, metastatic models of melanoma using an allograft system ^{8a, 8b}. Herein, we established a solid xenograft melanoma tumor at one primary site, which is ideal for assessing the effects of angiogenesis. In this melanoma model, the tumors are highly pigmented, allowing for ease of visualization and measurements on nude mice. Our shift away from the highly metastatic allograft model is due to its poor reflection of human disease along with dubious conclusions that might be drawn from biological data derived by using it ³⁷.

Through the solid xenograft melanoma model, we discovered that the mechanism of action of CVS-16 (**10b**) *in vivo* is the reduction of angiogenesis, which is not what we had predicted based on the data gleaned from our *in vitro* studies. Since we detected reduced viability in the presence of CVS-16 (**10b**), we hypothesized that CVS-16 (**10b**) had a direct effect against tumor cells, possibly an inhibition of mitogenesis. However, mitogenesis inhibition is not what we observed in animals, rather it was angiogenesis inhibition. Interestingly, the angiogenic response of ATX was previously described as “comparable to that elicited by VEGF” ^{13a}. Thus, our data is consistent with the known properties of ATX. Although we cannot completely rule out all other molecular mechanisms that may also contribute to this phenomenon, we can state that angiogenesis does not proceed *in vivo*, in the presence of 40-50 mg/kg CVS-16 (**10b**).

It is highly likely that 50 mg/kg (or very close to) is the MTD for CVS-16 (**10b**), certainly without pre-emptive supportive therapy for the animals. One treated mouse (50 mg/kg) died, presumably to the unspecified side effects of CVS-16 (**10b**) since it had only a miniscule-sized tumor and no obvious signs of metastases, even at necropsy. However, it is not uncommon to have 1 or 2 otherwise healthy, untreated mice in our animal colony die from dehydration for no obvious reason deduced by our veterinary staff. Nevertheless, the fact that a mouse possibly succumbed to side effects is consistent with the harsh adverse drug events manifested by angiogenesis inhibitors. For example, bevacizumab contains several black-box warnings, which includes: fatal hemorrhage, increased arterial thromboembolic events (myocardial infarction and stroke), gastrointestinal perforation and complications to wound healing which requires discontinuation of the drug at least 28 days before surgery³⁸. These black-box warnings are in addition to the other known drug toxicities of bevacizumab including hypertension, proteinuria and central nervous system events (e.g. dizziness, depression, headaches, seizure, lethargy, visual disturbances, etc.)³⁹.

Although adverse events are a concern for further pre-clinical development of ATX inhibitors, most anticancer therapeutics, in particular traditional, cytotoxic chemotherapy, produces harsh side effects for patients. However, this regrettable actuality is tolerated because of favorable therapeutic indexes and the potential for ameliorating a life-threatening illness. Even the newer classes of targeted biologics possess severe unwanted side effects, some even serving as a measure of therapeutic activity (e.g. an acne-like rash). Thus, there is extreme variability among patient compliance and tolerability of anticancer therapeutics, along with patients' willingness to continue therapy.

Although CVS-16 (**10b**) (40-50 mg/kg) was capable of sustaining an inhibition on tumor volume for approximately 53 days after the injection of tumor cells, the sizes of tumors in the highest treated group of animals started to increase on day 54. This was 6 days after we had reduced the dosage in this group from 50 mg/kg to 40 mg/kg. We cannot be certain whether this shift from static to growing tumors was due to the dosage reduction, chemoresistance or another factor. We did not observe any change in the amount of circulating VEGF among the treated mice in comparison to the controls (data not shown). This argues against a shift to VEGF or chemoresistance using this mechanism, which is what we had predicted since ATX works similarly to VEGF, and inhibiting both might be logically necessary to sustain a long-term response against tumor angiogenesis. Therefore, our supposition is that 50 mg/kg of CVS-16 (**10b**) is required for a sustained inhibition of ATX-dependent angiogenesis and tumor progression and that supportive care is required at this dosage.

Nevertheless, it was very exciting to observe the ability of CVS-16 (**10b**) to prevent melanoma tumor progression in animals. Advanced melanoma is a particularly difficult type of cancer to treat because it is unresponsive to traditional chemotherapy; therefore, immunotherapy is typically administered even though responses are achieved in less than 20% of patients. In the past few years, several new therapeutics for melanoma were approved for the first time in over a decade and these included trametinib, dabrafenib and vemurafenib. The drugs are intended for patients with activating BRAF mutations, which occurs in a majority of melanomas, but not all cases. Thus, more research is desperately

needed to uncover drugs that can treat melanoma, especially since the incidence of this disease is rising. Besides melanoma, the compound was also effective against the viability and migration of MDA-MB-231 breast cells *in vitro*. This is very intriguing considering this cell line was isolated from the pleural effusion of a patient and represents a highly invasive triple-negative breast cancer, which is a clinically challenging subtype to treat. Taken together, our data supports further pre-clinical testing of the vinyl sulfone CVS-16 (**10b**) as an ATX inhibitor in a combination approach with other anticancer therapeutics against the progression of melanoma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

This work was supported in part by a Research Scholar Grant 120634 from the American Cancer Society (to M.M.M.), NS 29632 (to G.D.P.), HL 070231 (to G.D.P.) and the Georgia Research Alliance (to M.M.M.). G.W.J. would like to thank Schering-Plough/Merck for financial support during conduct of this project. M.M.M. would like to thank Caitlin Ruddick and Santosh Patel for their work in the laboratory. The following authors contributed to the design of the research study (MMM, GWJ, DM, GDP), performed the research and collected data (MMM, GWJ, MKA, WJ, DTN, JMF, HTN, SKT, AAA, DM), analyzed the data (MMM, GWJ, MA, WJ, DTN, WJH, DM, GDP) and wrote the paper (MMM, GWJ, GDP).

GDP is a consultant for Echelon Biosciences, Inc; GWJ received financial support from Schering-Plough/Merck during the period this work was performed. DM worked at Echelon Biosciences, Inc. during the period this work was performed. GWJ currently works for a major hedge fund company in New York.

LIST OF ABBREVIATIONS

ATX	autotaxin
LPA	lysophosphatidic acid
LPC	lysophosphatidylcholine

REFERENCES

1. Umezū-Goto M, Kishi Y, Taira A, Hama K, Dohmae N, Takio K, Yamori T, Mills GB, Inoue K, Aoki J, Arai H. Autotaxin has lysophospholipase D activity leading to tumor cell growth and motility by lysophosphatidic acid production. *J Cell Biol.* 2002; 158(2):227–33. [PubMed: 12119361]
2. Murata J, Lee HY, Clair T, Krutzsch HC, Arestad AA, Sobel ME, Liotta LA, Stracke ML. cDNA cloning of the human tumor motility-stimulating protein, autotaxin, reveals a homology with phosphodiesterases. *J Biol Chem.* 1994; 269(48):30479–84. [PubMed: 7982964]
3. Stracke ML, Krutzsch HC, Unsworth EJ, Arestad A, Cioce V, Schiffmann E, Liotta LA. Identification, purification, and partial sequence analysis of autotaxin, a novel motility-stimulating protein. *J Biol Chem.* 1992; 267(4):2524–9. [PubMed: 1733949]
4. (a) 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. Autotaxin, a secreted lysophospholipase D, is essential for blood vessel formation during development. *Mol Cell Biol.* 2006; 26(13):5015–22. [PubMed: 16782887] (b) Tanaka M, Okudaira S, Kishi Y, Ohkawa R, Iseki S, Ota M, Noji S, Yatomi Y, Aoki J, Arai H. Autotaxin stabilizes blood vessels and is required for embryonic vasculature by producing lysophosphatidic acid. *J Biol Chem.* 2006; 281(35):25822–30. [PubMed: 16829511]

5. Fotopoulou S, Oikonomou N, Grigorieva E, Nikitopoulou I, Paparountas T, Thanassopoulou A, Zhao Z, Xu Y, Kontoyiannis DL, Remboutsika E, Aidinis V. ATX expression and LPA signalling are vital for the development of the nervous system. *Dev Biol.* 2010; 339(2):451–64. [PubMed: 20079728]
6. (a) Liu S, Murph M, Panupinthu N, Mills GB. ATX-LPA receptor axis in inflammation and cancer. *Cell Cycle.* 2009; 8(22):3695–701. [PubMed: 19855166] (b) Liu S, Umezu-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. Expression of autotaxin and lysophosphatidic acid receptors increases mammary tumorigenesis, invasion, and metastases. *Cancer Cell.* 2009; 15(6):539–50. [PubMed: 19477432]
7. Nakai Y, Ikeda H, Nakamura K, Kume Y, Fujishiro M, Sasahira N, Hirano K, Isayama H, Tada M, Kawabe T, Komatsu Y, Omata M, Aoki J, Koike K, Yatomi Y. Specific increase in serum autotaxin activity in patients with pancreatic cancer. *Clin Biochem.* 2011
8. (a) 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. Carba analogs of cyclic phosphatidic acid are selective inhibitors of autotaxin and cancer cell invasion and metastasis. *J Biol Chem.* 2006; 281(32):22786–93. [PubMed: 16782709] (b) 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. Targeting melanoma growth and viability reveals dualistic functionality of the phosphonothionate analogue of carba cyclic phosphatidic acid. *Mol Cancer.* 2010; 9:140. [PubMed: 20529378] (c) Singh AD, Sisley K, Xu Y, Li J, Faber P, Plummer SJ, Mudhar HS, Rennie IG, Kessler PM, Casey G, Williams BG. Reduced expression of autotaxin predicts survival in uveal melanoma. *Br J Ophthalmol.* 2007; 91(10):1385–92. [PubMed: 17475713]
9. 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. Serum autotaxin measurement in haematological malignancies: a promising marker for follicular lymphoma. *Br J Haematol.* 2008; 143(1):60–70. [PubMed: 18710386]
10. Kishi Y, Okudaira S, Tanaka M, Hama K, Shida D, Kitayama J, Yamori T, Aoki J, Fujimaki T, Arai H. Autotaxin is overexpressed in glioblastoma multiforme and contributes to cell motility of glioblastoma by converting lysophosphatidylcholine to lysophosphatidic acid. *J Biol Chem.* 2006; 281(25):17492–500. [PubMed: 16627485]
11. Tokumura A, Kume T, Fukuzawa K, Tahara M, Tasaka K, Aoki J, Arai H, Yasuda K, Kanzaki H. Peritoneal fluids from patients with certain gynecologic tumor contain elevated levels of bioactive lysophospholipase D activity. *Life Sci.* 2007; 80(18):1641–9. [PubMed: 17367815]
12. Samadi N, Gaetano C, Goping IS, Brindley DN. Autotaxin protects MCF-7 breast cancer and MDA-MB-435 melanoma cells against Taxol-induced apoptosis. *Oncogene.* 2009; 28(7):1028–39. [PubMed: 19079345]
13. (a) Nam SW, Clair T, Kim YS, McMarlin A, Schiffmann E, Liotta LA, Stracke ML. Autotaxin (NPP-2), a metastasis-enhancing motogen, is an angiogenic factor. *Cancer Res.* 2001; 61(18):6938–44. [PubMed: 11559573] (b) Ptaszynska MM, Pendrak ML, Stracke ML, Roberts DD. Autotaxin signaling via lysophosphatidic acid receptors contributes to vascular endothelial growth factor-induced endothelial cell migration. *Mol Cancer Res.* 2010; 8(3):309–21. [PubMed: 20197381]
14. Ptaszynska MM, Pendrak ML, Bandle RW, Stracke ML, Roberts DD. Positive feedback between vascular endothelial growth factor-A and autotaxin in ovarian cancer cells. *Mol Cancer Res.* 2008; 6(3):352–63. [PubMed: 18337445]
15. (a) Albers HM, Dong A, van Meeteren LA, Egan DA, Sunkara M, van Tilburg EW, Schuurman K, van Tellingen O, Morris AJ, Smyth SS, Moolenaar WH, Ovaa H. Boronic acid-based inhibitor of autotaxin reveals rapid turnover of LPA in the circulation. *Proc Natl Acad Sci U S A.* 2010; 107(16):7257–62. [PubMed: 20360563] (b) 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. A novel autotaxin inhibitor reduces lysophosphatidic acid levels in plasma and the site of inflammation. *J Pharmacol Exp Ther.* 2010; 334(1):310–7. [PubMed: 20392816] (c) Hoeglund AB, Bostic HE, Howard AL, Wanjala IW, Best MD, Baker DL, Parrill AL. Optimization of a pipemidic acid autotaxin inhibitor. *J Med Chem.* 2010; 53(3):1056–66. [PubMed: 20041668] (d)

Xu X, Yang G, Zhang H, Prestwich GD. Evaluating dual activity LPA receptor pan-antagonist/autotaxin inhibitors as anti-cancer agents in vivo using engineered human tumors. *Prostaglandins Other Lipid Mediat.* 2009; 89(3-4):140–6. [PubMed: 19682598] (e) Zhang H, Xu X, Gajewiak J, Tsukahara R, Fujiwara Y, Liu J, Fells JI, Perygin D, Parrill AL, Tigyi G, Prestwich GD. Dual activity lysophosphatidic acid receptor panantagonist/autotaxin inhibitor reduces breast cancer cell migration in vitro and causes tumor regression in vivo. *Cancer Res.* 2009; 69(13):5441–9. [PubMed: 19509223] (f) 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. S32826, a nanomolar inhibitor of autotaxin: discovery, synthesis and applications as a pharmacological tool. *J Pharmacol Exp Ther.* 2008; 327(3):809–19. [PubMed: 18755937] (g) Clair T, Koh E, Ptaszynska M, Bandle RW, Liotta LA, Schiffmann E, Stracke ML. L-histidine inhibits production of lysophosphatidic acid by the tumor-associated cytokine, autotaxin. *Lipids Health Dis.* 2005; 4:5. [PubMed: 15737239] (h) East JE, Kennedy AJ, Tomsig JL, De Leon AR, Lynch KR, Macdonald TL. Synthesis and structure-activity relationships of tyrosine-based inhibitors of autotaxin (ATX). *Bioorg Med Chem Lett.* 2010; 20(23):7132–6. [PubMed: 20951039] (i) Gupte R, Patil R, Liu J, Wang Y, Lee SC, Fujiwara Y, Fells J, Bolen AL, Emmons-Thompson K, Yates CR, Siddam A, Panupinthu N, Pham TC, Baker DL, Parrill AL, Mills GB, Tigyi G, Miller DD. Benzyl and Naphthalene Methylphosphonic Acid Inhibitors of Autotaxin with Anti-invasive and Anti-metastatic Activity. *ChemMedChem.* 2011(j) Prestwich GD, Gajewiak J, Zhang H, Xu X, Yang G, Serban M. Phosphatase-resistant analogues of lysophosphatidic acid: agonists promote healing, antagonists and autotaxin inhibitors treat cancer. *Biochim Biophys Acta.* 2008; 1781(9):588–94. [PubMed: 18454946] (k) 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. Synthesis, structure-activity relationships, and biological evaluation of fatty alcohol phosphates as lysophosphatidic acid receptor ligands, activators of PPARgamma, and inhibitors of autotaxin. *J Med Chem.* 2005; 48(15):4919–30. [PubMed: 16033271] (l) Albers HM, Ovaa H. Chemical evolution of autotaxin inhibitors. *Chem Rev.* 2012; 112(5):2593–603. [PubMed: 22335786] (m) St-Coeur PD, Ferguson D, Morin P Jr, Touaibia M. PF-8380 and closely related analogs: synthesis and structure-activity relationship towards autotaxin inhibition and glioma cell viability. *Arch Pharm (Weinheim).* 2013; 346(2):91–7. [PubMed: 23300119]

16. Ueda K, Yoshihara M, Nakao M, Tanaka T, Sano S, Fukuzawa K, Tokumura A. Evaluation of inhibitory actions of flavonols and related substances on lysophospholipase d activity of serum autotaxin by a convenient assay using a chromogenic substrate. *J Agric Food Chem.* 2010; 58(10):6053–63. [PubMed: 20415488]
17. van Meeteren LA, Ruurs P, Christodoulou E, Goding JW, Takakusa H, Kikuchi K, Perrakis A, Nagano T, Moolenaar WH. Inhibition of autotaxin by lysophosphatidic acid and sphingosine 1-phosphate. *J Biol Chem.* 2005; 280(22):21155–61. [PubMed: 15769751]
18. Nishimasu H, Okudaira S, Hama K, Mihara E, Dohmae N, Inoue A, Ishitani R, Takagi J, Aoki J, Nureki O. Crystal structure of autotaxin and insight into GPCR activation by lipid mediators. *Nat Struct Mol Biol.* 2011; 18(2):205–12. [PubMed: 21240269]
19. Hausmann J, Kamtekar S, Christodoulou E, Day JE, Wu T, Fulkerson Z, Albers HM, van Meeteren LA, Houben AJ, van Zeijl L, Jansen S, Andries M, Hall T, Pegg LE, Benson TE, Kasiem M, Harlos K, Kooi CW, Smyth SS, Ovaa H, Bollen M, Morris AJ, Moolenaar WH, Perrakis A. Structural basis of substrate discrimination and integrin binding by autotaxin. *Nat Struct Mol Biol.* 2011; 18(2):198–204. [PubMed: 21240271]
20. Powers JC, Asgian JL, Ekici OD, James KE. Irreversible inhibitors of serine, cysteine, and threonine proteases. *Chem Rev.* 2002; 102(12):4639–750. [PubMed: 12475205]
21. (a) Hanzlik RP, Thompson SA. Vinylogous amino acid esters: a new class of inactivators for thiol proteases. *J Med Chem.* 1984; 27(6):711–2. [PubMed: 6547487] (b) Mc Cormack T, Baumeister W, Grenier L, Moomaw C, Plamondon L, Pramanik B, Slaughter C, Soucy F, Stein R, Zuhl F, Dick L. Active site-directed inhibitors of *Rhodococcus* 20 S proteasome. Kinetics and mechanism. *J Biol Chem.* 1997; 272(42):26103–9. [PubMed: 9334174]
22. Bogyo M, McMaster JS, Gaczynska M, Tortorella D, Goldberg AL, Ploegh H. Covalent modification of the active site threonine of proteasomal beta subunits and the *Escherichia coli* homolog HsIV by a new class of inhibitors. *Proc Natl Acad Sci U S A.* 1997; 94(13):6629–34. [PubMed: 9192616]

23. Nguyen D, Nguyen O, Zhang H, Prestwich GD, Murph MM. Murph MM. A Bromophosphonate Analogue of Lysophosphatidic Acid Surpasses Dacarbazine in Reducing Cell Proliferation and Viability of MeWo Melanoma Cells. *Research on Melanoma - A Glimpse into Current Directions and Future Trends*. 2011 InTech.
24. Xu X, Prestwich GD. Inhibition of tumor growth and angiogenesis by a lysophosphatidic acid antagonist in an engineered three-dimensional lung cancer xenograft model. *Cancer*. 2010; 116(7): 1739–50. [PubMed: 20143443]
25. Ferguson CG, Bigman CS, Richardson RD, van Meeteren LA, Moolenaar WH, Prestwich GD. Fluorogenic phospholipid substrate to detect lysophospholipase D/autotaxin activity. *Org Lett*. 2006; 8(10):2023–6. [PubMed: 16671772]
26. (a) Luquin C, Sciorra VA, Morris AJ. Lysophosphatidic acid signaling: how a small lipid does big things. *Trends Biochem Sci*. 2003; 28(7):377–83. [PubMed: 12878005] (b) Moolenaar WH. Lysophosphatidic acid, a multifunctional phospholipid messenger. *J Biol Chem*. 1995; 270(22): 12949–52. [PubMed: 7768880]
27. North EJ, Osborne DA, Bridson PK, Baker DL, Parrill AL. Autotaxin structure-activity relationships revealed through lysophosphatidylcholine analogs. *Bioorg Med Chem*. 2009; 17(9): 3433–42. [PubMed: 19345587]
28. Lee Z, Swaby RF, Liang Y, Yu S, Liu S, Lu KH, Bast RC Jr. Mills GB, Fang X. Lysophosphatidic acid is a major regulator of growth-regulated oncogene alpha in ovarian cancer. *Cancer Res*. 2006; 66(5):2740–8. [PubMed: 16510595]
29. Loukinova E, Dong G, Enamorado-Ayalya I, Thomas GR, Chen Z, Schreiber H, Van Waes C. Growth regulated oncogene-alpha expression by murine squamous cell carcinoma promotes tumor growth, metastasis, leukocyte infiltration and angiogenesis by a host CXC receptor-2 dependent mechanism. *Oncogene*. 2000; 19(31):3477–86. [PubMed: 10918606]
30. Richmond A, Yang J, Su Y. The good and the bad of chemokines/chemokine receptors in melanoma. *Pigment Cell Melanoma Res*. 2009; 22(2):175–86. [PubMed: 19222802]
31. Jiang G, Xu Y, Fujiwara Y, Tsukahara T, Tsukahara R, Gajewiak J, Tigyi G, Prestwich GD. Alpha-substituted phosphonate analogues of lysophosphatidic acid (LPA) selectively inhibit production and action of LPA. *ChemMedChem*. 2007; 2(5):679–90. [PubMed: 17443831]
32. Jiang G, Madan D, Prestwich GD. Aromatic phosphonates inhibit the lysophospholipase D activity of autotaxin. *Bioorg Med Chem Lett*. 2011; 21(17):5098–101. [PubMed: 21489790]
33. (a) Parrill AL, Baker DL. Autotaxin inhibitors: a perspective on initial medicinal chemistry efforts. *Expert Opin Ther Pat*. 2010; 20(12):1619–25. [PubMed: 21047298] (b) Norman DD, Ibezim A, Scott WE, White S, Parrill AL, Baker DL. Autotaxin inhibition: development and application of computational tools to identify site-selective lead compounds. *Bioorg Med Chem*. 2013; 21(17): 5548–60. [PubMed: 23816044]
34. Saunders LP, Ouellette A, Bandle R, Chang WC, Zhou H, Misra RN, De La Cruz EM, Braddock DT. Identification of small-molecule inhibitors of autotaxin that inhibit melanoma cell migration and invasion. *Molecular cancer therapeutics*. 2008; 7(10):3352–62. [PubMed: 18852138]
35. Gupte R, Siddam A, Lu Y, Li W, Fujiwara Y, Panupinthu N, Pham TC, Baker DL, Parrill AL, Gotoh M, Murakami-Murofushi K, Kobayashi S, Mills GB, Tigyi G, Miller DD. Synthesis and pharmacological evaluation of the stereoisomers of 3-carba cyclic-phosphatidic acid. *Bioorg Med Chem Lett*. 2010; 20(24):7525–8. [PubMed: 21051230]
36. Parrill AL, Baker DL, Montedonico LE. Mechanism-based inactivators of autotaxin. 2011
37. Herlyn M, Fukunaga-Kalabis M. What is a good model for melanoma? *The Journal of investigative dermatology*. 2010; 130(4):911–2. [PubMed: 20231823]
38. Genentech, I. Avastin (bevacizumab) full prescribing information. Group, A. M. o. t. R., editor. South San Francisco: 2013. p. 26
39. Chu, E.; DeVita, VT. *Physicians' Cancer Chemotherapy Drug Manual*. Jones and Bartlett Publishers; Sudbury, MA: 2011. p. 591

Figure 1.

Synthesis of Quaternary Ammonium Vinyl Sulfone Analogs **10** (CVS-18, CVS-16).

Reagents and conditions: (a) K_2CO_3 , acetone, reflux, 2 h, 98%; (b) $TrCl$, triethylamine, dichloromethane, 94%; (c) $mCPBA$, dichloromethane, 0 °C to room temperature, 8 h, 95%; (d) NaH , RI , DMF, 72-88%; (e) $LiAlH_4$, diethyl ether, at 0 °C, 10 min, 34-51%; (f) TFA , dichloromethane, 2 h, 66-81%; (g) $MsCl$, $DIPEA$, dichloromethane; (h) NaI , acetone; (i) $DIPEA$, $EtOAc$, 45-81% for three steps; (j) trimethylamine in $EtOH$, dichloromethane, 4 days, 71-85%.

Figure 2.

Synthesis of Quaternary Ammonium Vinyl Sulfone Analogs **17** (CVS-12, CVS-6).

Reagents and conditions: (a) K_2CO_3 , acetone, reflux, 2 h, 89-93%; (b) $LiAlH_4$, THF, at 60 °C, overnight, 88-90%; (c) *m*-CPBA, dichloromethane, 0 °C to room temperature, 8 h, 77-81%; (d) $MsCl$, DIPEA, dichloromethane; (e) NaI , acetone; (f) DIPEA, EtOAc, 69-82% for three steps; (g) trimethylamine in EtOH, dichloromethane, 4 days, 58-72%.

Figure 3.

Synthesis of Non-quaternary ammonium Vinyl Sulfone **21 (PVS-16)**. Reagents and conditions: (a) NaH, C₁₆H₃₃I, DMF, 95%;; (b) LiAlH₄, Diethyl ether, -78 °C, 15 min, 33%; (c) MsCl, DIPEA, dichloromethane; (d) NaI, acetone; (e) NaOtBu, EtOAc/DCM, 77% for three steps.

Figure 4.

CVS-16 (**10b**) reduces cell viability in multiple cancer cell lines. Cell viability was measured using CellTiter® Blue after treating cells with the indicated concentration of CVS-16 (**10b**) for 48 h. The data is presented as a viability curve (A) and bar graph (B). * $p < 0.05$ comparing 0 μM to CVS-16-treated condition for each cell line using the student's *t*-test. The negative control, PVS-16 (**21**), a phenyl vinyl sulfone compound was used against multiple cancer cell lines. The data is presented as a viability curve (C) and bar graph (D).

Figure 5.

ATX inhibitors reduce the cell viability of MeWo melanoma cells, in contrast with chemotherapy. Cell viability was measured using CellTiter® Blue after treating cells with the indicated concentration of: CVS-16 (**10b**), HA-130, PF-8380, dacarbazine (DTIC), paclitaxel (PTX/TAX), vincristine (VCR) and cisplatin (CDDP) for 48 h. * $p < 0.05$ comparing CVS-16 (**10b**) to PF-8380-treated cells or ** $p < 0.01$ comparing CVS-16 (**10b**) to chemotherapy (DTIC, PTX/TAX, VCR and CDDP) using the student's *t*-test.

Figure 6.

CVS-16 (**10b**) blunts tumor progression by inhibiting angiogenesis in a melanoma xenograft model. (A) Animals with melanoma tumors were treated with the indicated concentrations of CVS-16 (**10b**) and established tumors were measured with calipers every other day. The arrow indicates the reduction of dosage from 50 mg/kg to 40 mg/kg on day 47 (see text for details). * $p < 0.05$ and *** $p < 0.001$, control (n=10) vs. 50-40 mg/kg (n=5). There was no significant difference among the control group (n=10) vs. 20 mg/kg (n=5). (B) Images of tumors on day 57 from control and treated animals. (C) Serum was collected and analyzed for KC/CXCL1. * $p < 0.05$, control vs. 20 mg/kg and *** $p < 0.001$, control vs. 50-40 mg/kg. (D) Quantification of specimens in the pathology report indicates the increase in tumor necrosis among animals treated with 50-40 mg/kg of CVS-16 (**10b**). ** $p < 0.01$, control vs. 40-50 mg/kg and * $p < 0.05$, 20 mg/kg vs. 40-50 mg/kg. (E) Analysis of tumor specimens for regions with endothelial cells present indicates differences among the groups. (F) ATX was measured from the serum of treated and control animals. * $p < 0.05$, comparing drug treated animals to controls using ANOVA followed by the Bonferroni multiple comparison test.

Figure 7.

The autotaxin inhibitors HA-130 and PF-8380 did not impact tumor progression in a xenograft model of melanoma. Animals with melanoma tumors were treated with the indicated concentrations of either: HA-130 (A), PF-8380 (B) or dimethylformamide (DMF) solvent control. The established pigmented tumors were measured with calipers every other day. (C) ATX was measured from the serum of treated and control animals.

Table 1

Inhibition of ATX phosphodiesterase activity by vinyl sulfone analogues.

Compound	R	K _i (μM)	95% CI
10a (CVS-18)	C ₁₈ H ₃₅	2.43	2.08-2.77
10b (CVS-16)	C ₁₆ H ₃₃	3.50	3.21-3.8
17a (CVS-12)	C ₁₂ H ₂₅	1.79	1.64-1.93
17b (CVS-6)	C ₆ H ₁₃	NE ^a	NA
21 (PVS-16)	C ₁₆ H ₃₃	NE ^a	NA

^a NE, no effect was shown at the highest concentration (10 μM) tested.

Table 2Inhibition of **10b** (CVS-16) in various cell lines.

10b		
Cell line	Average IC ₅₀ (μM)	95% CI
PC-3	1.93	0.91-4.45
OVCAR-3	4.44	2.53-8.10
HT-29	2.11	0.97-4.88
MeWo	4.08	2.87-5.79
MDA-MB-231	0.39	0.14-1.04
SB-2	4.89	2.73-9.12
SKOV-3	6.74	2.55-7.22