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Aromatic phosphonates inhibit the lysophospholipase D activity of autotaxin

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ABSTRACT

Autotaxin (ATX) is an attractive target for the anticancer therapeutics that inhibits angiogenesis, invasion and migration. ATX is an extracellular lysophospholipase D that hydrolyzes lysophosphatidylcholine to form the bioactive lipid lysophosphatidic acid. The aromatic phosphonate S32826 was the first described nanomolar inhibitor of ATX. However, the tridecylamide substituent on aromatic ring contributed to its poor solubility and bioavailability, severely limiting its utility in vivo. *c* Log *P* calculations revealed that the lipophilicity of S32826 could be lowered by shortening its hydrophobic chain and by introducing substituents alpha to the phosphonate. Herein, we describe the synthesis of a small set of α-substituted phosphonate analogs of S32826, and we show that shortening the chain and adding α-halo or α-hydroxy substituents increased solubility; however, ATX inhibition was reduced by most substitutions. An optimal compound was identified for examination of biological effects of ATX inhibition in vivo.

Autotaxin (ATX), a member of the ectonucleotide pyrophosphatase/phosphodiesterase 2 (NPP2 or ENPP2) family, is a secreted enzyme with extracellular lysophospholipase D activity.[1] ATX hydrolyzes lysophosphatidylcholine (LPC) to lysophosphatidic acid (LPA) and choline,[1,2] and is the predominant source of extracellular LPA. Thus, in addition to a normal functional role in stabilizing blood vessels during embryonic development, local production of LPA by ATX/lysoPLD could support the invasion of tumor cells, promoting metastasis.[3,4] The mechanisms of enhanced tumor cell invasion by LPA include two aspects. First of all, LPAR-mediated activation of the Rho and Rac GTPase pathways are essential for the regulation of the actin cytoskeleton and cell motility.[5] Second, LPA has been shown to regulate the activity of matrix metalloproteinases, which are also intimately involved in metastasis as well as in the LPA-induced transphosphorylation of the epidermal growth factor (EGF) receptor.[6] Thus, ATX inhibitors may have potential in cancer therapy by blocking the growth-supporting and anti-apoptotic effects of LPA and decreasing its titer.[7]

LPA and sphingosine-1-phosphate (S1P), which are the products of ATX-catalyzed hydrolysis of its substrates LPC and sphingosyl phosphorylcholine (SPC), respectively, were subsequently determined to show product feedback inhibition of ATX activity.[8] A number of substrates ATX inhibitors have been reported in recent years, including lysophosphatidic acid analogs,[7,9,10] phosphatidic acid derivatives,[11] Darmstoff analogs[12] and carbocyclic phosphatidic acid analogs.[13,14] Each of these ATX inhibitors was a phospholipid analog and possessed a phosphate, thiophosphate or phosphonate polar head attached to an alkyl or acyl moiety.

More recently non-lipid ATX inhibitors[15] have been identified via virtual screening[16] and pharmacophore dissection.[17] The newest inhibitors with in vivo efficacy include a boronic acid derivative[18] and an *N,N*-disubstituted piperidine.[19] During the preparation of this manuscript, two structures of ATX from rat[20] and from mouse[21] were reported, and undoubtedly this will further accelerate the discovery of ATX inhibitors.

In 2008, the aromatic phosphonate S32826 was described as the first reported nanomolar inhibitor of ATX, identified using high-throughput screening[22] (Fig. 1). The polar head of S32826 featured a metabolically-stabled phosphonate, similar to the α-substituted methylene phosphonate analogs of LPA.[7,10] The linker moiety of LPA was replaced by a phenyl ring in S32826, which restricted the flexibility of the linker compared to other LPA-analog ATX inhibitors. Despite its high potency, the poor solubility of S32826, which could be attributed to its high lipophilicity, limited its use in vivo. Such low solubility can cause low bioavailability or give rise to large fluctuations in the fraction absorbed in humans, and often cannot be compensated by a high membrane permeability. Furthermore, low solubility may be associated with stability problems and difficulties in developing an acceptable formulation.[23]

To address the low solubility of S32826, we synthesized a limited set of analogs in order to increase solubility while maintaining in vitro ATX inhibition. To this end, we reduced length of the amide chain and introduced polar substituents α to the phosphonate.

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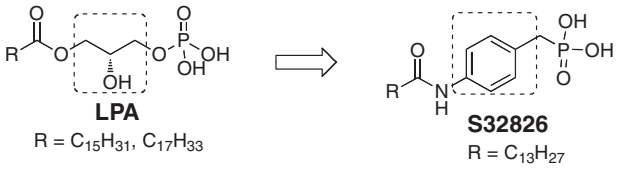


Figure 1. LPA and S32826.

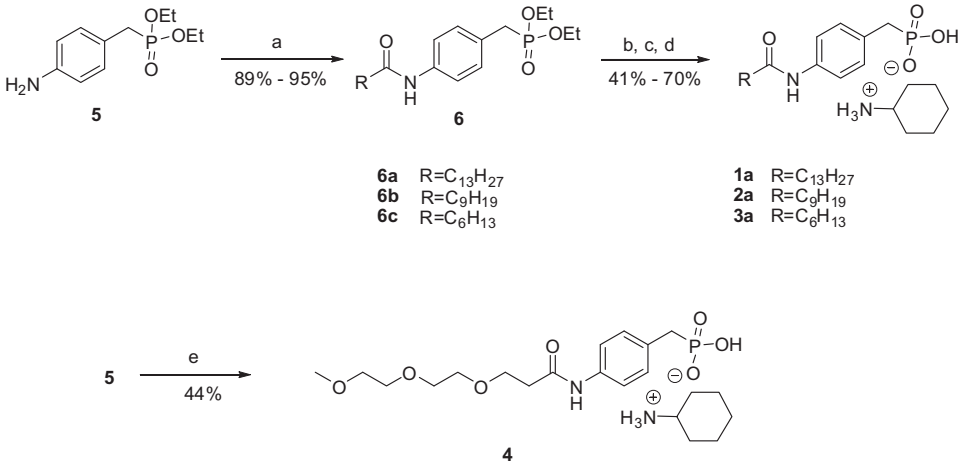
As illustrated in Scheme 1, each of the syntheses of the unsubstituted methylene phosphonate analogs **1a**, **2a**, **3a**, and **4** was completed using a three-step sequence. The acylation of commercially available phosphonate **5** proceeded smoothly and quantitatively to give the intermediate **6** by using acyl chloride in the presence of *N*,*N*-diisopropylethylamine at 0 °C. A clean conversion was observed by TLC, with a less polar spot emerging as starting material was consumed during the 2 h reaction. Treatment of the monoacylated diethyl phosphate **6** with trimethylsilyl bromide (TMSBr) in methylene chloride followed by hydrolysis with aqueous CH_3OH afforded the desired free acid. The final target compounds **1a**, **2a** and **3a** were precipitated out as cyclohexylammonium salts from the above solution by addition of cyclohexylamine. Then the resulting salts were washed with CH_3OH to give the final products as white solids. The oligo(ethylene glycol) analog **4** was synthesized by condensation of corresponding acid with phosphonate **5**. The final products were characterized by ^{1}H, ^{13}C, ^{31}P NMR and by HRMS. The ^{31}P spectrum of final product phosphonic acids displayed lower field signal (18.7 ppm) compared to their precursor phosphonates (26.4 ppm).

Exploration of additional diversity at the polar head position of S32826 was enabled as described in Schemes 2 and 3. The α-hydroxymethylene phosphonates are generally obtained by the Pudovik reaction,[24] which involves the nucleophilic addition of a dialkyl phosphite to a carbonyl compound in the presence of base. As shown in Scheme 2, aldehyde **7** was phosphorylated using a typical Pudovik procedure with diethyl phosphite and Et_3N to give the α-hydroxymethylene phosphonate **8** in good yield. In the ^{1}H NMR, a doublet at 5.15 ppm verified the presence of the α-hydroxymethylene function. Hydrogenation of the nitro group was not feasible due to the sensitivity of the benzylic hydroxyl group. Thus, the nitro group was reduced performed with Zn dust and ammonium chloride in a mixture of tetrahydrofuran, ethanol and water at 80 °C for 30 min to afford aniline **9** in excellent yield. The doublet at 4.86 ppm in the ^{1}H NMR confirmed that α-hydroxyl group had been retained in the molecule. The amides **10** and the cyclohexylammonium salts of compounds **1b**, **2b**, and **3b** were prepared from aniline **9** in an analogous fashion described previously for analog **1a**.

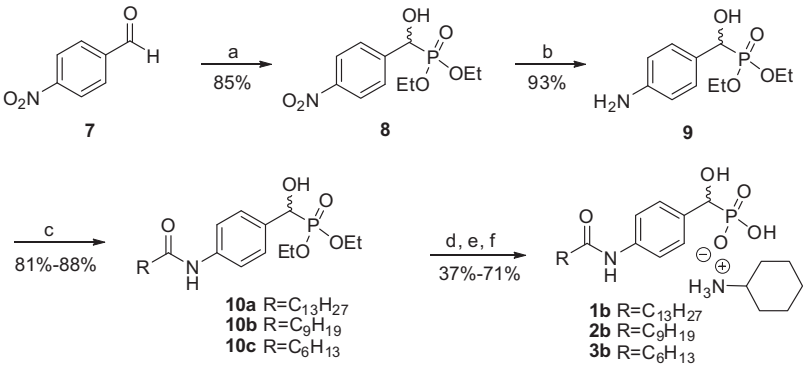
Scheme 3 shows the syntheses of the α-halomethylene phoshponate analogs. Nucleophilic displacement of hydroxyl with diethylaminosulfur trifluoride (DAST) in anhydrous methylene chloride gave the corresponding monofluorinated compounds **11** in moderate yield.[25] The ^{19}F NMR spectrum of **1c**, **2c** and **3c** had a doublet of doublet at −198.0 ppm with coupling constants of 89.5 Hz and 44.7 Hz, which was split by the hydrogen and phosphorus, respectively, confirming the existence of α-fluoro in the molecule. The α-bromomethylene phosphonates **12** were thus prepared by the reaction of the corresponding α-hydroxymethylene phosphonate with carbon tetrabromide and triphenylphosphine in methylene chloride. Subsequently, the final targets **1c**, **2c**, **3c**, and **1d** were prepared from the substituted phosphonate by using the protocols described above.

ATX inhibition was measured using FS-3, a fluorescence-quenched, lysophosphatidylcholine analog that reports directly on lysophospholipase activity[26] (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. DMSO was spiked into each reaction mixture to equalize vehicle concentrations. Recombinant, human ATX purified from Sf9 insect cells was employed as the enzyme source. Compounds were pre-incubated with the enzyme at 25 °C for 10 min, after which the fluorogenic substrate FS-3 was added. The rate of fluorescence increase was measured between 5 and 25 min after substrate addition; the fluorescence increase was linear during this time window. Rates were normalized to control reactions that contained all reaction components except the ATX inhibitor being tested.

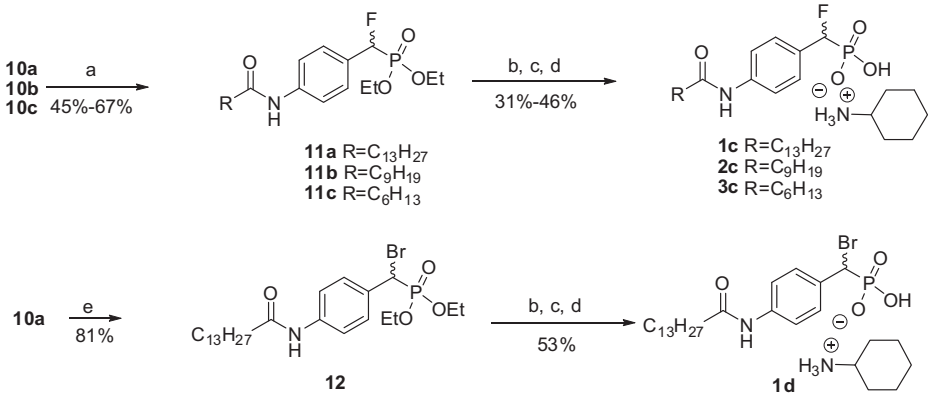
The results reported in Table 1 indicate that the binding affinity for the ATX decreases with the reduction of acyl chain length. The tetradecanoyl amide derivatives ($R = C_{13}H_{27}$) show the highest affinity. The standard compound **1a** displays an inhibitory potency (K_i = 9.0 nM) similar to that reported previously using different assay conditions.[22] Further optimization was attempted on changing the polar head from methylene phosphonate to α-substituted phosphonate. The α-fluoromethylene phosphonate analog **1c** was the most potent (K_i = 6.1 nM) ATX inhibitor in this series and α-bromomethylene phosphonate **1d** also showed very potent inhibitory activity (K_i = 8.1 nM). The *c* log *P* for each of the tetradecanoyl amide analogs exceeded 5.0, which led to poor aqueous solubility. The most encouraging compounds were the decanoyl



Scheme 1. Synthesis of methylene phosphonate analogs **1a**, **2a**, **3a** and **4**. Reagents and conditions: (a) RCOCl, DIPEA, dichloromethane, 2 h at 0 °C; (b) TMSBr, dichloromethane, overnight at rt; (c) CH_3OH/H_2O, 1 h at rt; (d) cyclohexylamine (e) acid, BopCl, dichloromethane.

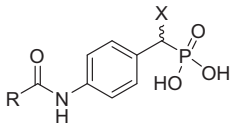


Scheme 2. Synthesis of α-hydroxymethylene phosphonate analogs **1b**, **2b** and **3b**. Reagents and conditions: (a) diethyl phosphite, triethylamine, dichloromethane, overnight at rt; (b) Zn, NH_4Cl, THF/EtOH/H_2O;(c) RCOCl, DIPEA, dichloromethane, 2 h at 0 °C; (d) TMSBr, dichloromethane, overnight at rt; (e) CH_3OH/H_2O, 1 h at rt; (f) cyclohexylamine.



Scheme 3. Synthesis of α-halomethylene phosphonate analogs **1c**, **2c**, **3c** and **1d**. Reagents and conditions: (a) DAST, dichloromethane, 2 h at 0 °C then rt; (b) TMSBr, dichloromethane, overnight at rt; (c) CH_3OH/H_2O, 1 h at rt; (d) cyclohexylamine; (e) CBr_4, PPh_3, dichloromethane, overnight at rt.

Table 1
Inhibition of autotaxin (ATX) phosphodiesterase activity by S32826 analogs



Compound	R	X	K_i (nM)	*c* Log *P*[a]
1a	$C_{13}H_{27}$	H	9	6.01
1b	$C_{13}H_{27}$	OH	24	5.42
1c	$C_{13}H_{27}$	F	6.1	5.88
1d	$C_{13}H_{27}$	Br	8.1	6.54
2a	C_9H_{19}	H	18	3.9
2b	C_9H_{19}	OH	24.2	3.31
2c	C_9H_{19}	F	72	3.77
3a	C_6H_{13}	H	512	2.3
3b	C_6H_{13}	OH	1009	1.72
3c	C_6H_{13}	F	834	2.18
4	OEG[b]	H	NE[c]	−0.96

[a] *c* Log *P* was calculated by using ChemBioDraw Ultra11.0
[b] OEG = $CH_3O(CH_2)_2O(CH_2)_2O(CH_2)_2$.
[c] NE, no effect observed at 10 μM.

amide derivatives (R = C_9H_{19}). Methylene phosphonate analog **2a** and α-hydroxymethylene phosphonate analog **2b** exhibited slightly reduced ATX inhibition (K_i = 18.0 nM and 24.2 nM). In contrast, the *c* log *P* showed a two log unit decrease, indicating that **2a** and **2b** would show improved aqueous solubility. This predicted trend of water solubility was evident when solutions of these compounds were prepared. The solubility of **2b** in water was 4 mg/mL compared to 1 mg/mL for **1a**. Lastly, the compounds modified with the shortest acyl chain (heptanoyl amide) showed much reduced potency (K_i = 0.5–1 μM range). Hence, we conclude there is a balance between the chain length and efficacy. Additionally, the compound **5** with oligo(ethylene glycol) acyl chain was very water soluble, but did not show any inhibitory activity at the highest concentration tested (10 μM).

In summary, we developed a set of S32826 analogs in order to increase solubility while maintaining ATX inhibitory potency. In contrast to the poor solubility of **1a**, α-hydroxymethylene phosphonate analog **2b** (R = C_9H_{19}) was identified as compound with potential in vivo utility, and displayed good potency and acceptable solubility. The use of this compound in preclinical models will be described elsewhere in due course.

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