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The chemical synthesis of metabolically stabilized 2-OMe-LPA analogues and preliminary studies of their inhibitory activity toward autotaxin

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ABSTRACT

The chemical synthesis of five new metabolically stabilized 2-*OMe*-LPA analogues (**1a**-**e**) possessing different fatty acid residues has been performed by phosphorylation of corresponding 1-*O*-acyl-2-*OMe*-gly-cerols which were prepared by multistep process from racemic glycidol. The now analogues were subjected to biological characterization as autotaxin inhibitors using the FRET-based, synthetic ATX substrate FS-3. Among tested compounds 1-*O*-oleoyl-2-*OMe*-LPA (**1e**) appeared to be the most potent, showing ATX inhibitory activity similar to that of unmodified 1-*O*-oleoyl-LPA. Parallel testing showed, that similar trend was also observed for corresponding 1-*O*-acyl-2-*OMe*-LPA (**1e**) was found to be resistant toward alkaline phosphatase as opposed to unmodified 1-*O*-oleoyl-LPA.

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Numerous studies conducted over last decades by several research groups have shown that lysophospholipids are not only structural components of cellular membranes but they also show important biological activity, influencing a broad variety of metabolic processes. Their prominent representatives, lysophosphatidic acids (LPA) are potent modulators of many hallmarks of cancer including cell proliferation, survival, migration, invasion, and neovascularization. A major step towards understanding of the biological activities of LPA was made by the unexpected discovery that the lysophospholipase D, the enzyme responsible for biosynthesis of LPA in serum was identical with autotaxin (ATX, also known as NPP2), a widely expressed nucleotide pyrophosphatase/phosphodiesterase (NPP).^{1,2} There are several lines of evidence that ATX is capable of producing not only LPA, but also cyclic phosphatidic acids (cPA) containing a dioxaphospholane ring spanning the sn-2 and sn-3 positions of glycerol.³

Considering the fact that autotaxin is coded by one of the 40 most upregulated genes in invasive cancers, ATX became an attractive pharmacological target.⁴ Following the discovery of ATX' feedback inhibition by LPA, several groups have reported the synthesis of metabolically stabilized analogues of LPA and cPA that could lead to novel anticancer chemotherapies.⁵ However, a limited number of ATX inhibitors with LPA structure have been reported to date.^{6–9} To address the need of longer biological half-lives, the

phosphate group was replaced with charged or neutral phosphoromimetics including methylene phosphonates, α -X methylene phosphonates (where X equals F, Br or OH), phosphorothioates or phosphonothioates.¹⁰ These modifications make such analogues resistant against lysophospholipid phosphatases (LPP). Recently, we have also described the chemical synthesis of new sulfur analogues of lysophospholipids, including phosphorothioate/phosphorodithioate derivatives of 2-OMe-LPA and phosphorothioate/ phosphorodithioate derivatives of CPA.¹¹ The most potent lipidbased inhibitor S32826 was described in 2008.¹² Its polar head featured a metabolically-stable phosphonate, similar to the α -substituted methylene phosphonate analogues of LPA. Subsequently, the α -fluoromethylene, α -bromomethylene, and hydroxymethylene phosphonate analogues of S32826 were described to increase low solubility of the parent molecule.¹³ However, numerous non-lipid inhibitors have been also reported and very recently they have been amply reviewed by Albers and Ovaa.¹⁴ Such small molecules targeting ATX activity include boronic acid-based derivatives,^{15,16} and an *N*,*N*-disubstituted piperidine.¹⁷ The latter inhibitors have a long linear and flexible structure, which is also reflected in the structure of LPA and LPC.

In this report, we describe the chemical synthesis of a series of 2-*OMe*-LPA analogues **1a–e** (without sulfur), unable to undergo $1\rightarrow 2$ acyl migration by anchoring the acyl chain in the 1 position of glycerol. We have subjected them to biological characterization as autotaxin inhibitors using the FRET-based, synthetic ATX substrate FS-3. Among tested compounds 1-*O*-oleoyl-2-*OMe*-LPA appeared to be the most potent. Since one major catabolic fate of LPA is the hydrolysis of its phosphate head group by ubiquitous

Abbreviations: ATX, autotaxin; cPA, cyclic phosphatidic acid; LPA, lysophosphatidic acid; NMR, nuclear magnetic resonance; MS, mass spectrometry.

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2-*OMe*-LPA analogues **1a-e** (X=O) 2-*OMe*-phosphorothioate-LPA analogues **2a-e** (X=S) R-C(O)- : **a**-lauroyl, **b**-myristoyl, **c**-palmitoyl, **d**-stearoyl, **e**-oleoyl

Figure 1. Structure of 2-OMe analogues of LPA (1a-e) and their phosphorothioate congeners (2a-e).



Scheme 1. Synthesis of 2-OMe analogues of LPA (1a-e).

phosphatases, we have additionally shown resistance of our analogues to the action of alkaline phosphatase.¹⁸ In addition, the autotaxin inhibitory properties of **1a–e** were compared with those of previously synthesized¹¹ phosphorothioate derivatives of 2-*OMe*-LPA (**2a–e**).

For the studies of inhibition of autotaxin, five new 2-OMe-LPA analogues (**1a**–**e**) were synthesized, possessing in *sn*-1 position the residues of lauric, myristic, palmitic, stearic and oleic acid, respectively (Fig. 1). Thus, a series of compounds was synthesized, bearing five different fatty acid residues both saturated (12:0, 14:0, 16:0, 18:0) and unsaturated (18:1). Similarly, an analogous series of phosphorothioate derivatives of 2-OMe-LPA (**2a**–**e**) was synthesized as described earlier.¹¹ The concept of 'metabolic stabilization' by introduction of 2-OMe group was invented by the research team that synthesized 1-O-oleoyl-2-OMe-sn-glycerophosphorothioate (OMPT) and studied its interactions with various LPA receptors.¹⁹

Table 1		
Inhibition of autotaxin (A	ΓX) activity by LF	PA analogues

The first steps of the synthesis were realized as described in our previous report starting from racemic glycidol (Scheme 1).¹¹ Thus, glycidol was incubated for 5 h at ca. 85 °C with equimolar amounts of corresponding fatty acids in the presence of catalytic quantity of tri-*n*-butylamine.²⁰ Crude 1-*O*-acylglycerols **3a–e** were crystallized from ether to give chromatographically pure products in 42–68% isolated yield. The functionalization of 1-*O*-acylglycerols was achieved by selective protection of primary hydroxyl function in the presence of a secondary one with *tert*-butyldimethylsilyl group (TBDMS) in 42–78% yield.²¹ The resulting 3-*O*-silyl ethers were further methylated at 2-hydroxyl group with trimethylsilyldiazomethane (TMSCHN₂) in the presence of 40% aqueous fluoroboric acid (HBF₄).²² The TBDMS group was removed by standard method employing *tetra*(*n*-butyl)ammonium fluoride (TBAF) in THF solution²¹ to give 1-*O*-acyl-2-*OMe*-glycerols **4a–e** in 64–92% yield.¹¹

Compounds **4a-e** were phosphitylated with N.N-diisopropylamino-0.0-(bis)-2-cvanoethylphosphoramidite²³ in the presence of S-ethylthiotetrazole, to give, after standard water-iodine oxida-1-O-acyl-2-OMe-3-O,O-(bis)-2-cyanoethylphosphorylglycetion. rols 5a-e in 65-73% yield. The O-2-cyanoethyl groups were removed by the action of DBU on **5a-e**, after its silvlation with *N*,*O*-bis(trimethylsilyl)acetamide (BSA).²⁴ The final 1-O-acyl-2-OMe-glycerol-3-O-phosphates (1a-e) were isolated as ammonium salts in 18-23% yield, after ion-exchange on Amberlyst[®]-NH₄⁺ resin. The compounds **1a-e** were isolated after lyophilization in a form of white solids, and were stable when stored at -20 °C. They were found to be soluble in alcohols (methanol, ethanol) and in water. The ammonium phosphates **1a-e** and all intermediate compounds were isolated by silica gel flash chromatography (FC). Their purity and identity was confirmed by TLC, NMR (¹H and ³¹P) and mass spectrometry (MALDI TOF and HR ESI). The details are given as Supplementary data. The series of 1-O-acyl-2-OMe-glycerol-3-O-phosphorothioates (2a-e) was synthesized from 4a-e by oxathiaphospholane approach as described in detail in our previous paper.11

Compounds **1a–e** and **2a–e** were examined for their ability to block ATX-mediated hydrolysis of lysophosphatidylcholine analogue FS-3 as the substrate, using an assay based on fluorescence resonance energy transfer (FRET). The ATX activity was measured in the presence of the lysophospholipid analogues added under different concentrations and was compared with the ATX inhibitory effects of unmodified LPA (18:1) and α -bromomethylene phosphonate LPA (BrP-LPA). The ATX activity measured without LPA analogues was used as the standard (100% activity). Time courses of FS-3 hydrolysis were linear over the time scale examined (60 min). The results showed that all of the 2-OMe-LPA analogues (1a-e) inhibited ATX activity to some extent (Table 1). Comparing ATX preferences for hydrophobic tail length (12:0, 14:0, 16:0, 18:0) and degree of unsaturation (18:0 vs 18:1) the least potent 2-OMe-LPA analogues appeared to be those containing lauroyl and stearoyl acyl chains, 1a and 1d (IC₅₀ = 1200.6 nM and

Compound	Chemical structure	Inhibition (%) at 1 µM conc. ±SD	$IC_{50} \pm SD(nM)$
1a	1-O-lauroyl-2-OMe-LPA	45.5 ± 1.5	1200.6 ± 72.7
1b	1-O-mirystoyl-2-OMe-LPA	57.7 ± 1.7	704.5 ± 79.5
1c	1-O-palmitoyl-2-OMe-LPA	74.5 ± 7.1	361.1 ± 41.8
1d	1-O-stearoyl-2-OMe-LPA	49.7 ± 0.6	1012.8 ± 25.4
1e	1-O-oleoyl-2-OMe-LPA	81.3 ± 6.7	220.2 ± 36.0
2a	1-O-lauroyl-2-OMe-thioLPA	36.7 ± 1.8	2410.1 ± 452.2
2b	1-O-mirystoyl-2-OMe-thioLPA	62.3 ± 1.8	595.8 ± 49.9
2c	1-O-palmitoyl-2-OMe-thioLPA	64.7 ± 0.4	512.0 ± 66.6
2d	1-O-stearoyl-2-OMe-thioLPA	35.5 ± 1.3	2418.0 ± 70.2
2e	1-O-oleoyl-2-OMe-thioLPA	77.8 ± 11.5	355.6 ± 79.5
BrP-LPA	BrP-LPA	86.1 ± 1.6	170.3 ± 10.4
1-O-Oleoyl-LPA	1-O-Oleoyl-LPA	65.6 ± 1.7	499.8 ± 68.2

1012.8 nM, respectively). The same preference was demonstrated in the case of corresponding sulfur analogues (2a and 2d), however their inhibitory potency was even lower ($IC_{50} = 2410.1$ nM and 2418.0 nM, respectively). The most significant ATX inhibition $(IC_{50} = 220.2 \text{ nM})$ was observed for **1e** (1-O-oleoyl-2-OMe-LPA). It was comparable with that of known ATX inhibitors, α-bromomethylene phosphonate LPA (BrP-LPA) and unmethylated 1-O-oleoyl-LPA.⁶ Replacement of one phosphate oxygen atom with sulfur (**2e**) resulted in a slightly higher IC_{50} value (355.6 nM).

Recent studies have demonstrated that several ectoenzymes located on the extracellular side of the cell plasma membrane catalyze hydrolysis of LPA thus limiting its potential usefulness as drugs. The degradation of extracellular LPA can be mainly attributed to the ectophosphatase activity of plasma membrane lipid phosphate phosphatases (LPPs). The second LPA conversion pathway involves the action of lysophosphatidic acid acyltransferases (LPAAT) catalyzing the transfer of an acyl group from acyl-CoA to LPA to form phosphatidic acid (PA).¹⁰ It was hypothesized that thiophosphate substitution may confer resistance to LPP-mediated degradation in the case of the ester-linked thiophosphate derivative (1-oleoyl-2-O-methyl-rac-glycerophosphothionate, OMPT).^{19a} It is known that the rate of hydrolysis of a phosphate ester by alkaline phosphatase is independent of ester group and for example, nucleoside 5'-phosphorothioates are degraded 2000 times slower than the corresponding phosphates.²⁵ It was also proved that another phosphorothioate synthetic analogue of LPA, octadecenyl thiophosphate (OTP) was resistant to LPP1 phosphatase activity derived from mouse embryonic fibroblasts.²⁶ Recently, we have also described the chemical synthesis of new sulfur analogues of lysophospholipids, including phosphorothioate/phosphorodithioate derivatives of 2-OMe-LPA.¹¹ Therefore, we have examined unmodified 1-O-oleoyl-LPA, α-bromomethylene phosphonate LPA and the most active 1-O-acyl-2-OMe-derivative of LPA (1e) for their resistance toward degradation by alkaline phosphatase isolated from bovine intestinal mucosa. We have measured the amount of inorganic phosphate liberated from the substrate according to modified phosphomolybdate blue assay.²⁷ Unfortunately, we couldn't apply this method to 1-O-oleovl-2-OMe-phosphorothioate (2e) since inorganic phosphorothioate does not form a blue ammonium molybdate complex.²⁵ Unmethylated 1-O-oleoyl-LPA was completely dephosphorylated after 24 h of incubation at 37 °C with the enzyme. However, 1-O-oleoyl-2-OMe-LPA (1e) was found to be completely resistant toward alkaline phosphatase under identical conditions, similarly to BrP-LPA. These observations suggest that introduction of methyl group into sn-2 position of LPA may also protect LPA analogues against the action of lysophospholipid phosphatases in vivo.

In summary, our studies demonstrate that the most significant ATX inhibition was observed for 1-O-oleoyl-2-OMe-LPA (1e), and this compound was also resistant to the action of alkaline phosphatase what makes it the best candidate as autotaxin inhibitor among 2-OMe-LPA analogues studied.

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Supplementary data

Supplementary data (experimental procedures, characterization data) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.03.008.

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