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PII: S0960-894X(16)30573-X
DOI: http://dx.doi.org/10.1016/j.bmcl.2016.05.075
Reference: BMCL 23934

To appear in: *Bioorganic & Medicinal Chemistry Letters*

Received Date: 23 March 2016
Revised Date: 25 May 2016
Accepted Date: 26 May 2016

Please cite this article as: Sowińska, A., Rytczak, P., Gendaszewska-Darmach, E., Drzazga, A., Koziołkiewicz, M., Okruszek, A., The chemical synthesis and preliminary biological studies of phosphodiester and phosphorothioate analogues of 2-methoxy-lysophosphatidylethanolamine, *Bioorganic & Medicinal Chemistry Letters* (2016), doi: http://dx.doi.org/10.1016/j.bmcl.2016.05.075

The chemical synthesis and preliminary biological studies of phosphodiester and phosphorothioate analogues of 2-methoxy-lysophosphatidylethanolamine

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The chemical synthesis of phosphorothioate/phosphodiester analogues of 2-methoxy-lysophosphatidylethanolamine has been described. For the preparation of phosphorothioate derivatives oxathiaphospholane approach has been employed. The phosphodiester compounds were prepared by OXONE® oxidation of corresponding phosphorothioates. Each lysophospholipid analogue was synthesized as a series of four compounds, bearing different fatty acid residues both saturated (14:0, 16:0, 18:0) and unsaturated (18:1). The methylation of glycerol 2-hydroxyl function was applied in order to increase the stability of prepared analogues by preventing 1->2 acyl migration. The cytotoxicity of newly synthesized 2-methoxy-lysophosphatidylethanolamine derivatives was evaluated with resazurin-based method in prostate cancer PC3 cell line. The highest reduction of cell viability was noted for LPE analogues containing myristoyl acyl chain.

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For many years lysophospholipids (LPLs) have been considered as membrane components functioning as mediators in the pathways of the synthesis of various phospholipids and as structural components embedding proteins into cell membranes. However, the rapidly expanding chemistry and biology of lipids revealed that lysophospholipids also act as intercellular lipid mediators and can be regarded as hormone-like signaling molecules.[1] Primary representatives of this broad family are lysophosphatidic acid (LPA), sphingosine-1-phosphate (S1P), and lysophosphatidylcholine (LPC). While LPA, S1P or even LPC have been studied in detail, the actions of other LPLs such as lysophosphatidylethanolamine (LPE) have not been investigated to such a degree.

Natural 1-*O*-acyl-*sn*-glycerol-3-*O*-phosphoethanolamine (lysophosphatidylethanolamine, LPE, **1**) has a zwitterionic structure (see Fig. 1) and exists in optically active form (R enantiomer), as a mixture of several compounds, possessing fatty acids of different length and saturation status (16:0, 18:0, 18:1,18:2, 20:1, 20:3, 20:4, 22:6). LPE accumulates in human serum reaching concentration of several hundreds ng/mL.[2]

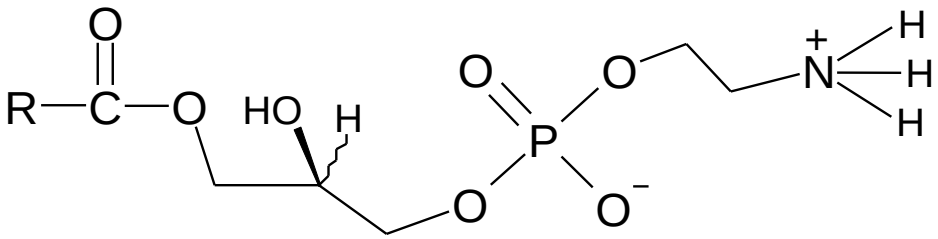


Figure 1. The structure of natural R-lysophosphatidylethanolamine (LPE, **1)**. Substituents R-C(O)- refer to various fatty acid residues present in lysophospholipids.

Informations concerning LPE as a bioactive lipid are rather sparse as compared to other phospholipids. 16:0 LPE together with taurocholic acid, and 22:5 LPC have been defined as "marker metabolites" that can be used to distinguish the different stages of hepatocarcinogenesis.[3] Regarding carcinogenesis, LPE was found to induce intracellular calcium mobilization in OVCAR-3 and SK-OV3 human ovarian cancer cells as well as chemotactic migration and cellular invasion in SK-OV3 cells.[4] Lysophosphatidylethanolamine can also increase intracellular Ca^{2+} concentration as well as cellular proliferation and migration in MDA-MB-231 breast cancer cells.[5] Furthermore, LPE from mushroom *Grifola frondosa* extract inhibited serum deprivation-induced apoptosis and induced increase in intracellular Ca^{2+} concentration and neuronal differentiation in PC-12 neuronal cells.[6]

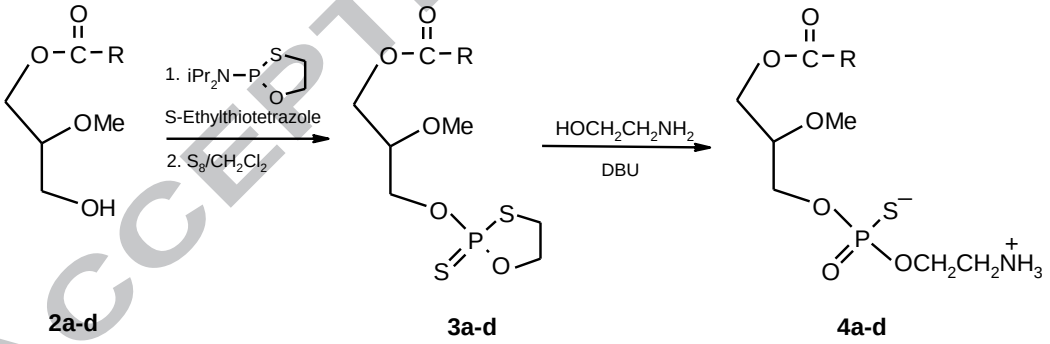
In our previous publications we have described the chemical synthesis of sulfur analogues of LPA, cPA and LPC, in which either one or two nonbridging phosphate oxygen atoms are substituted by sulfur to give phosphorothioate or phosphorodithioate analogues, respectively.[7-9] In addition, for LPA and LPC analogues also 2-hydroxyl function was modified by its methylation. In many studies regarding lysophospholipids an attention of researchers was paid mainly to S1P, LPA, cPA and LPC containing oleoyl or palmitoyl fatty acid residues as representatives of two types LPL with unsaturated or saturated carbon chains. Our recent studies have emphasized the significance of the sulfur and acyl chain length on the biological activity of LPC[10]. These results as well as intriguing biological properties of natural LPE prompted us to undertake studies on the synthesis of its analogues possessing different acyl groups and modified both within phosphate group and at carbon 2 of glycerol. On the basis of our earlier studies performed on the synthesis of phosphorothioate derivatives of nucleotides and oligonucleotides[11] we expected that lysophospholipid phosphorothioate analogues should have similar physicochemical properties as natural lysophospholipids, yet should be more resistant towards hydrolytic enzymes.[12] In order to prevent possible 1->2 acyl migration in LPE analogues, the oxygen atom at position 2 of glycerol was protected by methylation.[7-9,13,14] In fact this modification should also increase the metabolic stability of both phosphorothioate and phosphodiester analogues of LPE. The conditions of chemical synthesis allow us also to obtain LPE analogues as homogenic compounds, each containing only one fatty acid residue. Thus, 1-acyl-2-methoxy-LPE phosphorothioateanalogues (**4a-d**) were prepared as a series of four

different compounds, bearing the residues of the following fatty acids: **a**) myristic (14:0), **b**) palmitic (16:0), **c**) stearic (18:0),**d**) oleic (18:1).

The diversity of biological functions and activity of lysophosphatidylcholine allowed us to assume that LPE phosphorothioate/phosphodiester derivatives **4** and **5** may also have interesting biological or even therapeutic properties. For preliminary biological studies the LPE phosphorothioates (**4a-d**) and phosphodiesters (**5a-d**) were synthesized starting from racemic glycidol.

The chemical synthesis of 1-acyl-2-methoxyglycerols (**2a-d**), that were crucial building blocks for the preparation of **4a-d** was performed exactly as described in our previous papers.[7-9] Thus, all four aforementioned fatty acids were reacted with commercially available racemic glycidol in the presence of catalytic amounts of *n*-tributylamine into 1-acylglycerols according to described procedure.[15] In the following step 1-acylglycerols were regioselectively silylated with *t*-butyldimethylsilyl chloride in the position 3 of glycerol (primary hydroxyl group).[16] The silyl ethers were then methylated at the central oxygen of glycerol with trimethylsilyldiazomethane in the presence of 40% fluoroboric acid.[17] The 2-methoxy ethers were desilylated with tetra-*n*-butylammoniumfluoride in tetrahydrofuran,[16] yielding desired glyceride building blocks **2a-d**. All aforementioned products were isolated and purified by silica gel flash column chromatography and characterized by spectroscopic methods as described earlier.[7-9]

The introduction of one nonbridging sulfur atom into lysophosphatitydylethanolamine molecule was performed using oxathiaphospholane approach.[18] This method was successfully employed for the synthesis of phosphorothioate nucleotide and oligonucleotide analogues. Its application for the synthesis of phosphorothioate analogues of 1-acyl-2-methoxy-lysophosphatidylethanolamine **4a-b** is shown on Scheme 1.



Scheme 1. The synthesis of LPE 1-acyl-2-methoxy-phosphorothioates (**4a-d**)
Acyl groups: **a** – myristoyl, **b** – palmitoyl, **c** – stearoyl, **d** – oleoyl; yield 56-83%

For the synthesis of phosphorothioate analogues racemic 1-acyl-2-methoxyglycerols **2a-d** were reacted in anh. dichloromethane solution with equimolar amount of 2-*N,N*-diisopropylamino-1,3,2-oxathiaphospholane in the presence of *S*-ethylthiotetrazole, and then with elemental sulfur, to give 3-(2-thio-1,3,2-oxathiaphospholane) lipid derivatives **3a**-**d**, each showing in ^{31}P NMR two signals at δ *ca* 105 ppm. Such chemical shifts were earlier described as characteristic for various 2-alkoxy-2-thio-1,3,2-oxathiaphospholane derivatives.[18] Compounds **3a-d** were isolated by silica gel flash column chromatography as pale-yellow oils and characterized by spectroscopic methods. Due to appearance of two

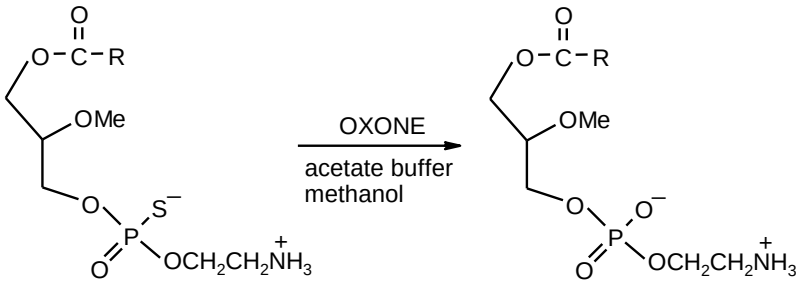
signals in ^{31}P NMR spectra it was concluded that each of **3a-d** is a mixture of two stereoisomers, that however could not be separated by flash chromatography or TLC. Each of oxathiaphospholane derivatives **3a**-**d** was then treated in anh. dichloromethane with 3 molar equivalents of ethanolamine and 2 molar equivalents of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU). The ring opening condensation reaction occurred exclusively at hydroxyl group of ethanolamine[19] and was accompanied by spontaneous elimination of episulfide.[18] Crude products were purified by silica gel flash column chromatography in chloroform/methanol/water gradient to give LPE 1-acyl-2-methoxy-phosphorothioates (**4a-d**) as white solids in 56-83% yield. Each product was obtained as a mixture of two stereoisomers that was indicated by the presence of two signals in ^{31}P NMR at δ ca 59 ppm. Such chemical shifts are characteristic for dialkyl phosphorothioates.[11,18] The purified products **4a-d** were characterized by spectroscopic methods (^{1}H and ^{31}P NMR, MALDI TOF MS). They were soluble in chloroform, methanol and water, and did not decompose when stored at -20^{o}C. The details are given in Table 1 and in supplementary material.

Table 1. Yields, ^{31}P NMR and MS data of LPE 1-acyl-2-methoxy-phosphorothioates **4a-d**

Compound	^{31}P NMR ($CDCl_3$) [ppm]	MALDI TOF MS (M^-) [m/z]	Calculated MW [Da]	Yield [%]
4a	59,85; 59,92	454,4	455,61	83
4b	59,93; 60,01	482,4	483,66	61
4c	59,19; 59,26	510,3	511,71	56
4d	58,42; 58,50	508,5	509,70	82

The presence of two signals in ^{31}P NMR spectra of each **4a-d** (and also **3a-d**) can be attributed to the presence of two centers of asymmetry (at C2 and at phosphorus), leading to the formation of two pairs of diastereoisomers. Unfortunately, these stereoisomers could not be separated by either column or thin layer chromatography (TLC).

For the synthesis of phosphodiester LPE analogues each of racemic 1-acyl-2-methoxy-LPE phosphorothioates **4a-d** containing one of the aforementioned fatty acid residues were reacted with an excess of OXONE® in methanol/acetate buffer (pH 5.6) by stirring for 18 h at 38^{o}C (see Scheme 2). The commercially available OXONE® reagent is a mixture of potassium salts ($2KHSO_5xKHSO_4xK_2SO_4$) containing potassium peroxymonosulphate with known oxidizing properties. Its application for the oxidation of various phosphorothioates into corresponding phosphates was recently described in the literature.[20]



4a-d **5a-d**

Scheme 2. The synthesis of LPE 1-acyl-2-methoxy-phosphodiesters (**5a-d**)
Acyl groups: **a** – myristoyl, **b** – palmitoyl, **c** – stearoyl, **d** – oleoyl; yield 12-22%

Crude products were purified by silica gel flash column chromatography to give 1-acyl-2-methoxy-LPE phosphodiesters **5a-d** as white solids in 12-22% yield. Compounds **5a-d** showed in ^{31}P NMR single signals at δ ca 0 ppm, characteristic for dialkyl phosphodiesters.[21] The purified products **5a-d** were characterized by spectroscopic methods (^{1}H and ^{31}P NMR, LSI MS). They were soluble in chloroform, methanol and water, and were found to be stable on storage at -20^{o}C. The details are given in Table 2 and in supplementary material. The products were obtained as enantiomeric mixtures.

Table 2. Yields, ^{31}P NMR and MS data of 1-acyl-2-methoxy-LPE phosphodiesters **5a-d**

Compound	^{31}P NMR ($CDCl_3$) [ppm]	LSI MS (M^-) [m/z]	Calculated MW [Da]	Yield [%]
5a	0,61	438,3	439,61	13
5b	0,68	466,4	467,66	12
5c	0,70	494,4	495,71	18
5d	0,02	492,5	493,70	22

The chemical synthesis of both phosphodiester[21,22] and phosphorothioate[23] derivatives of phosphatidylethanolamine, containing two fatty acid residues at positions 1 and 2 of glycerol, has been described in literature with application of corresponding 3-oksazaphospholidine intermediates. However, this methodology appeared to be ineffective for the synthesis of aforementioned 1-acyl-2-methoxyl phosphodiester and phosphorothioate analogues of lysophosphatidylethanolamine (data not shown).

All newly synthesized compounds **4a-d** and **5a-d** were subjected to biological characterization for their *in vitro* cytotoxic activity in human prostate PC3 cancer cell line (PC3), which is known to express LPA1 receptor[24] utilized not only by LPA but also by LPE.[5] The cells (5,000 per well) were seeded onto 96-well plates and cultured for 24 and 48 h, followed by treatment with modified LPE derivatives. Viability of treated PC3 cells was examined using a resazurin-based PrestoBlue cell viability reagent. The cytotoxicity data are presented in Figures 2 and 3. All of the tested compounds, both at 5 μM and at 10 μM concentration did not significantly inhibit PC3 viability. The exception was 10 μM 1-myristoyl-2-methoxy-LPE (**5a**) reducing the number of viable cells to 68.2% and 59.8% after 24- and 48 hour treatment, respectively (Fig. 2).

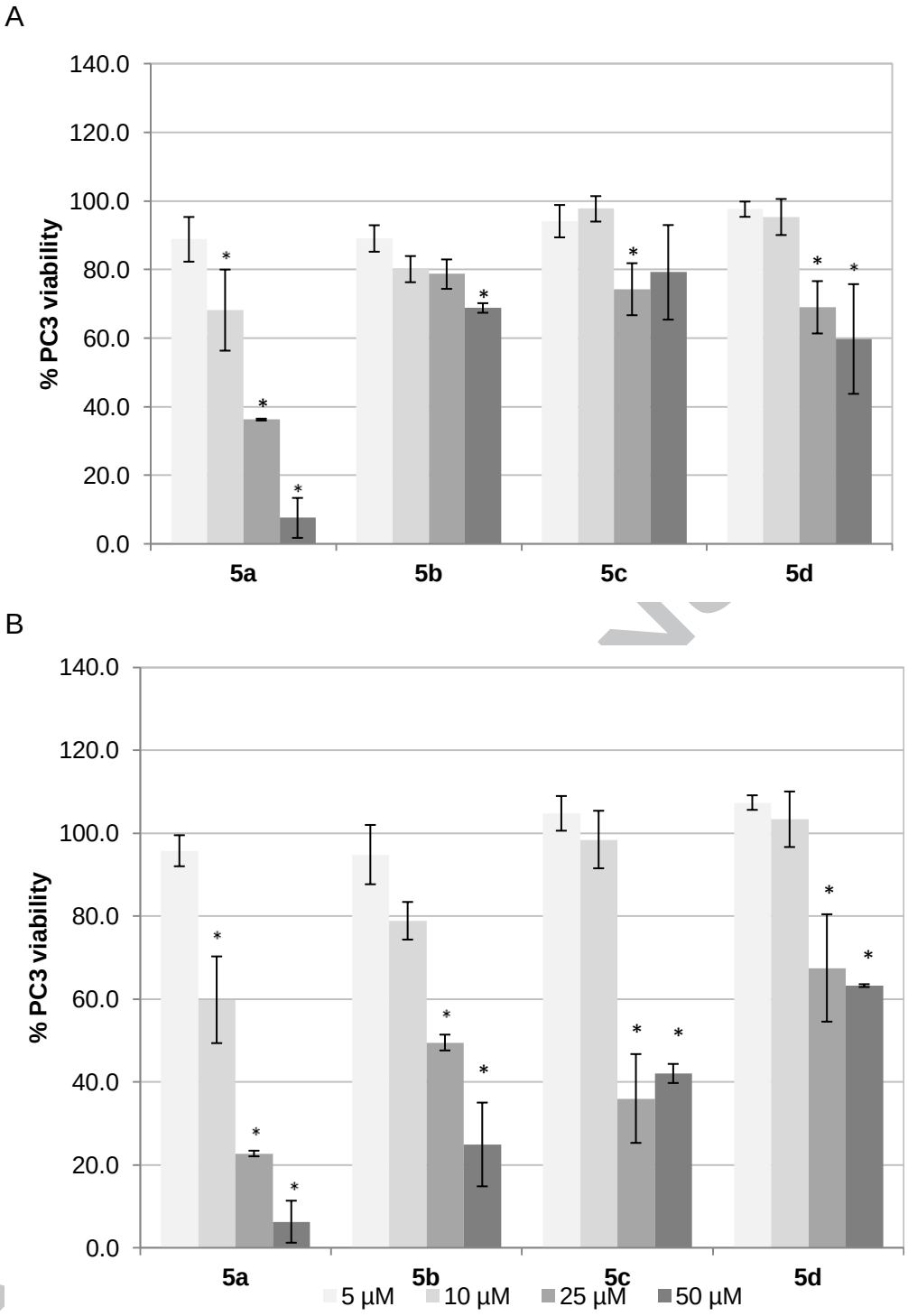


Figure 2. The effect of 1-acyl-2-methoxy-LPC phosphodiester analogues **5a-d** on the viability of prostate cancer PC3 cells. Viability was assessed using PrestoBlue kit after 24 h (**A**) and 48 h (**B**) of incubation. Data represent the means ± SD from at least three independent experiments; *P < 0.05, versus unstimulated control cells (100 %)

Compound **5a** appeared to be the most toxic also at higher concentration used (25 µM and 50 µM), reducing the cellular viability to 22.7% and 6.3% as compared to untreated control cells after 48 hours. 1-Palmitoyl-2-methoxy-LPE (**5b**) appeared to be the second among tested LPLs with regard to its cytotoxicity. After 48 h incubation **5b** reduced the number of cells to 78.8%, 49.5% and 24.9% when applied at the 10 µM, 25 µM and 50 µM

concentration, respectively. Significant cytotoxic effect was also observed for other species without sulfur (**5c** and **5d**), however the effect was lower than in the case of **5a** and **5b**. It is noteworthy that all phosphorothioate analogues appeared to be less toxic as compared to their phosphodiester counterparts (Fig. 3).

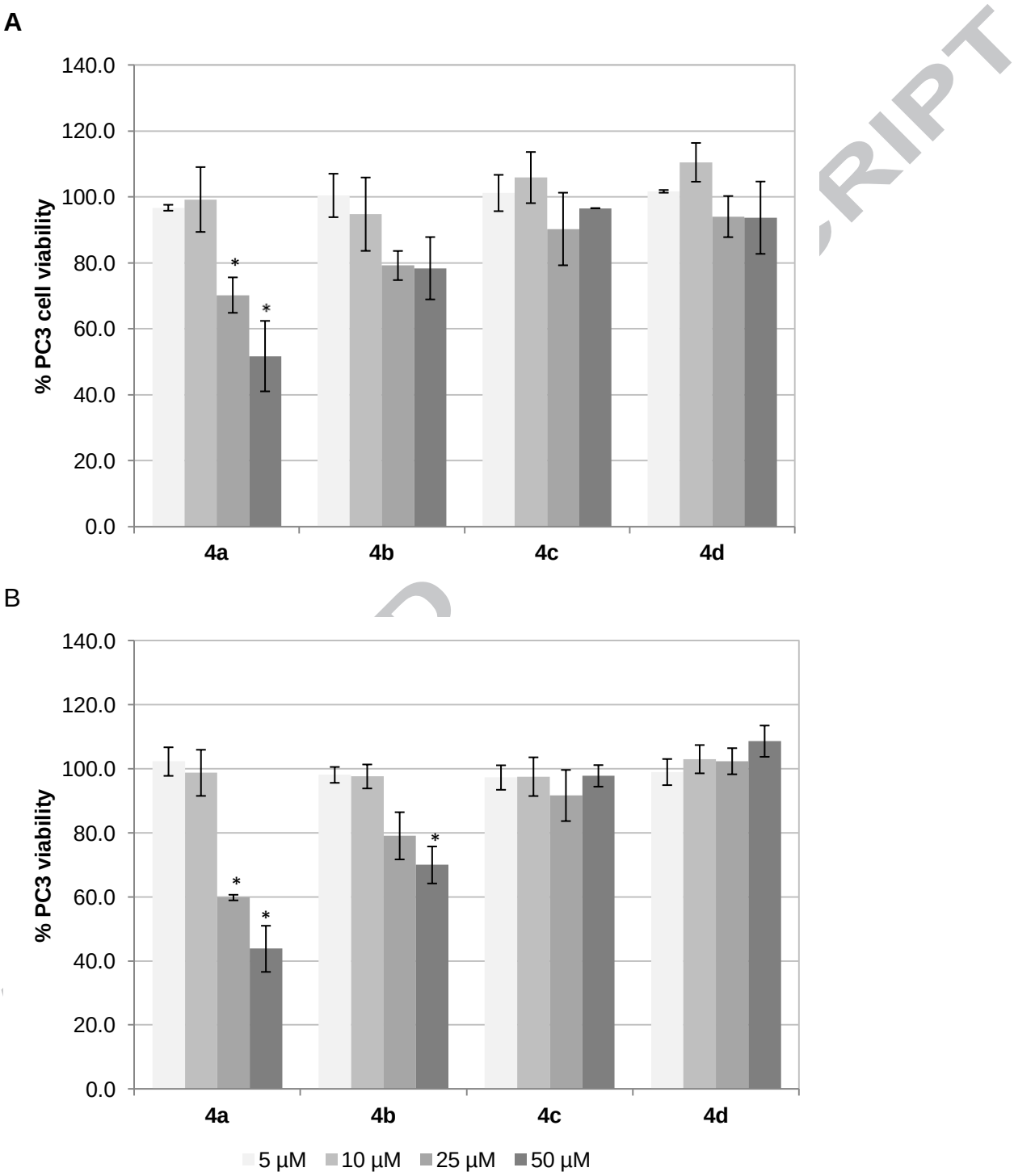


Figure 3. The effect of 1-acyl-2-methoxy-LPC phosphorothioate analogues **4a-d** on the viability of prostate cancer PC3 cells. Viability was assessed using PrestoBlue kit after 24 h (**A**) and 48 h (**B**) of incubation. Data represent the means ± SD from at least three independent experiments; *P < 0.05, versus unstimulated control cells (100 %)

1-Oleoyl-2-methoxy-lysophosphatidylethanolamine phosphorothioate (**4d**) and 1-stearoyl-2-methoxy-lysophosphatidylethanolamine phosphorothioate (**4c**) did not exhibit any cytotoxicity even at the 50 μM concentration. Other sulfur-containing compounds decreased the number of viable cells after 48 h to 59.8% and 43.8% in the case of 1-mirystoyl-2-methoxy-lysophosphatidylethanolamine phosphorothioate (**4a**) and to 79.1% and 70.0% for 1-palmitoyl-2-methoxy-lysophosphatidylethanolamine phosphorothioate (**4b**) used at the 25 μM and 50 μM concentration, respectively.

In summary, almost all tested compounds showed low toxicity against PC3 prostate cancer cells when used at the 5 μM and 10 μM concentration. On the other hand, only 1-stearoyl-2-methoxy-lysophosphatidylethanolamine phosphorothioate (**4c**) and 1-oleoyl-2-methoxy-lysophosphatidylethanolamine phosphorothioate (**4d**) appeared to be non-toxic even at the 50 μM concentration. Surprisingly, unlike in case of LPCs, the highest reduction of cell viability was noted for LPE analogues containing myristoyl acyl chain. The myristoyl LPC analogues were found by us to be much less toxic than corresponding palmitoyl derivatives.[9,10] Similar effect was also observed in neutrophils, where stimulation with 1-palmitoyl-LPC induced calcium flux with modest membrane permeabilization.[25]

The new phosphorothioate analogues of 1-acyl-2-methoxy-LPE were prepared employing oxathiaphospholane chemistry.[18] It was found that their oxidation with OXONE® can provide corresponding LPE phosphodiester analogues. For each analogue a series of four compounds was synthesized, containing different fatty acid residues with both saturated (14:0, 16:0, 18:0) and unsaturated (18:1) hydrocarbon chains. For preliminary biological studies, the LPE analogues were prepared from racemic glycidol. Thus, LPE phosphorothioate analogues were obtained as a mixture of diastereoisomers and phosphodiester analogues were enantiomers. The attempts to synthesize the most active myristoyl compounds in stereodefined form at C2 carbon will be performed in the next step of research starting from enantiomers of glycidol.

Recent studies on chemistry and biology of lysophospholipids revealed that these compounds are important intercellular lipid mediators and can be regarded as signaling molecules.[1] However, further development of research focused on molecular functions of LPL and their receptors requires search of highly specific agonists and antagonists. Our methods allow to synthesize wide range of new, glycerol-based LPL analogues with unique structures and properties different from their native counterparts. These compounds were found to be valuable tools in the studies on the LPL mechanism of action.[8-10]

The main goal of our previous studies has been the chemical synthesis of LPL analogues (LPA, cPA, LPC) modified by the presence of methoxyl group in *sn*-2 position of glycerol skeleton (LPA, LPC) and presence or absence of phosphorothioate function within hydrophilic head group.[2b,7-10] In the course of those studies, followed by preliminary biological investigations, we have noted that important factor influencing biological activities of cPA and LPC analogues is acyl chain length and geometry. However, one should take into account that the concept of research regarding this issue has been neglected in many cases; the best documented studies on activities of cPA or LPC species concern only 18:1 (oleoyl) and 16:0 (palmitoyl) derivatives. Significant cytotoxicity was observed only in the case of 16:0 LPC analogues, while other derivatives appeared to be much less toxic.[10] We suggested that this effect might be caused by some nonspecific interactions of palmitoyl

LPC with cell membrane and its partial permeabilization.[10] In earlier studies of other authors I was observed that LPC 16:0 was more active in ROS generation than other LPC with different length of fatty chain.[26] Moreover, oleoyl LPC with unsaturated acyl chain (18:1) couldn't produce reactive oxygen species.[26]

Taking into account these observations we decided to synthesize LPE analogues with four different acyl chains: 14:0, 16:0, 18:0 and 18:1.They were supposed to exhibit the similar cytotoxic profile as LPC due to high structure similarity. Unexpectedly, the highest reduction of PC3 cell viability was observed for LPE analogues containing myristoyl acyl chain unlike in the case LPC where palmitoyl-containing derivative was found to be most toxic. For comparison, the cytotoxicity data of corresponding 1-acyl-2-methoxy-LPC analogues (phosphodiesters and phosphorothioates) obtained in the same cell system are presented in supplementary material. These results do not confirm abovementioned nonspecific damage of cell membrane and indicate an existence of unknown factor(s) that discriminate LPE and LPC containing different acyl chains.

It was demonstrated that unsaturation of the acyl chain makes the compound less-toxic (in the case of phosphodiester LPE analogues) or non-toxic (in the case of phosphorothioate LPE analogues) as compared to the cytotoxicity of other LPE analogues. Although specific LPE receptors have not been identified, Im et al. suggested that membrane LPA1 receptor can be activated not only by LPA but also by LPE. [4,5b] On the other hand, some results of his group indicate no involvement of LPA1 receptor in LPE-induced cellular response.[4,5b] We suggest that highest cytotoxicity of myristoyl LPE derivatives as compared to other LPE analogues cannot be mediated by the single LPA1 receptor. A recently resolved crystal structure of LPA1 indicates that the receptor can recognize a diverse set of ligands with different lengths and geometry of acyl chains rather than being specific to the commonly used oleoyl LPA (18:1) and probably cannot discriminate between myristoyl-, palmitoyl- and oleoyl-LPE.[27] Therefore, factors discriminating structurally related LPE remain to be identified.

Cytotoxicity of sulfur-containing LPE analogues **4a-d** appeared to be lower than those observed for corresponding phosphodiester compounds **5a-d**. Similar effects have been observed in our earlier studies for the corresponding LPC analogues.[9,10] This unexpected result should be further analyzed by molecular modeling methods and/or physicochemical approach.

Acknowledgement

This work was supported by a grant from the Polish National Science Centre (grant no. 2011/01/B/ST5/06383).

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Graphical abstract

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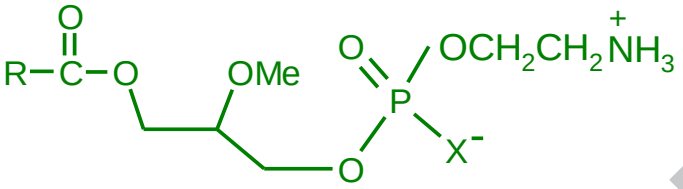

Phosphodiesters (X=O); Phosphorothioates (X=S)

2-OMe-LPE phosphorothioates (myristoyl, palmitoyl, stearoyl and oleoyl derivatives) were prepared by oxathiaphospholane method; their oxidation with OXONE® led to corresponding phosphodiester analogues. The products were tested for their cytotoxicity toward prostate cancer PC3 cell line.