Orginal Research Article

Synthesis of liponucleotides using bacterial phospholipase D

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GRAPHICAL ABSTRACT

5'-Phosphatidylclofarabine

R_1 and R_2 - alkyls of fatty acid residues
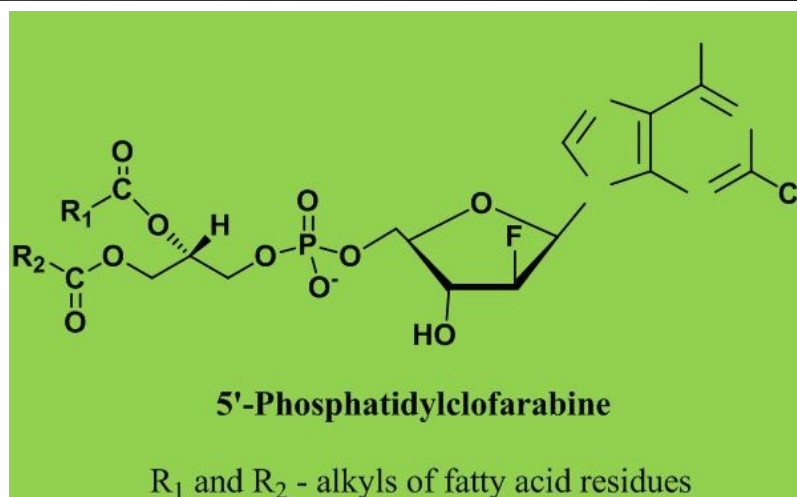
KEYWORDS

ABSTRACT

A series of conjugates of pharmacologically promising modified nucleosides with phospholipid was synthesized. Some liponucleotides were originally produced. Soybean lecithin served as the donor of phosphatidyl residue. Phospholipase D from strain *Streptomyces netropsis* BIM B-428D was engaged as biocatalyst in transphosphatidylation reaction. The yield of liponucleotide synthesis reaction varied in the range 44–95 mol% depending on the acceptor of phosphatidyl residue. 65 mg (about 68 μmoles) of pure phosphatidylclofarabine was recovered by chromatography on silica gel column, resulting in 68 mol% yield calculated from the amount of modified nucleoside supplied into the reaction mixture.

1- Introduction

Drugs based on modified nucleosides and nucleotides play a crucial role in chemical therapy of cancer and severe viral infections [1-3]. The list of pharmaceuticals encompassing well-known and recognized agents (5-fluorouridine, 2-chloro-2'-deoxyadenosine, 2-fluoroadenine arabinoside, azidothymidine, virazole (ribavirin) is constantly replenished with new entries (clofarabine, nelarabine, etc). Previous years have seen the emergence of novel clinically promising medicines combining the unique properties and principally distinct mechanism of action.

One of the latest papers [4] described and experimentally substantiated the radically innovative approach to treatment of cancer diseases leading to the selective self-elimination of tumor cells. It is based on the ability of the enzyme telomerase to insert 6-thio-2'-deoxyguanosine (modified analog of natural 2'-deoxyguanosine) into telomerase structure. The inclusion of modified nucleoside drastically alters the composition of chromosome terminus so that it loses the capacity to be shielded by protective proteins, triggering as a consequence cancer cell apoptosis and death [5].

Recently riboside of phytohormone kinetin was proposed as the effective cancerostatic compound. It was shown that kinetin riboside displayed therapeutic efficiency in regard to acute myeloblast leucosis [6] and anti-proliferative activity toward colorectal cancer cells [7]. Cytotoxic effects of kinetin riboside are determined by the ability to cause rapid ATP exhaustion provoking genotoxic stress wherein gene *CDKNIA* and other shock-involved genes are activated.

Clofarabine (2-chloro-2'-fluoradenine arabinoside) is referred to modern highly active antitumor nucleoside preparations. Clofarabine is primarily used for treatment of grave child leukemia cases not susceptible to therapy by other kinds of drugs [8-10]. Preclinical trials also revealed the potential of clofarabine to suppress growth of various solid tumors.

It is well known, however, that the majority of compounds suitable for oncotherapy suffer from side effects and limitations, like low curative index, marked toxic effects, rapid catabolism in blood vessels down to inactive substances, restraining thereby their clinical application. One of the key solutions to overcome this problem is elaboration of novel generation of pharmaceuticals (the so-called pro-drugs) by conjugating antiviral and antitumor nucleosides with phospholipids [11-13]. The resulting conjugates are distinguished by increased bio-accessibility, enhanced stability in blood stream, upgraded pharmacokinetic parameters, inferior toxicity. It was found, for instance, that certain dialkylglycerophosphate derivatives of clofarabine showed lower toxicity than the parent compound but preserved the elevated antitumor activity [14]. Intraperitoneal or per-oral administration of diacylglycerophosphates of clofarabine and fludarabine resulted in prolonged secretion of active nucleosides and their long-term retention in blood circulation system of test animals.

The available chemical methods of phospholipid conjugation with nucleosides are complicated and are characterized by small yields of the end products [14, 15]. Earlier our team demonstrated the possibility of using relatively simple and effective

biotechnology devised by the Japanese researchers [16] for the synthesis of phospholipid derivatives of a series of natural and modified nucleosides [17, 18]. This technique envisages application of bacterial phospholipase D.

Phospholipase D (PLD, EC 3.1.4.4) is the enzyme catalyzing hydrolysis of phosphodiester bonds in phospholipids to yield phosphatidic acid and alcohol residue. In addition to hydrolytic activity, PLD also may catalyze substitution of polar phospholipid groups in the course of the process termed transphosphatidylation [16-20] (**Fig. 1**).

Thus, mentioned above, most of the studied PLDs, apart from hydrolysis reaction, are able to transfer phosphatidyl residue to the primary or, under special conditions, to the secondary alcohol group of various compounds. It should be noted that PLD recovery from plants and microorganisms coupled to sufficiently high efficiency of transphosphatidylation reaction make this method appropriate for production of diverse phospholipids in preparative amounts. To illustrate this statement, the readily available phosphatidylcholine was used as the substrate to derive phosphatidylethanolamine [21], phosphatidylserine [22], phosphatidylglycerol [23] and less distributed phospholipids – 6-phosphatidyl-L-ascorbate [24], vitamin E derivatives [25], phosphatidylnucleosides [16, 26, 27], phospholipid-phytosterols [28] and a number of other compounds. The synthesized phospholipids may find use as emulsifiers, ingredients of cosmetic formulas, medicines and liposomal preparations [29].

Aim of this study – production of new phosphatidyl derivatives of pharmacologically valuable modified nucleosides using PLD from *Streptomyces netropsis* culture.

2. Materials and Methods

The modified nucleosides: 2-fluoroadenine arabinoside (fludarabine), 2-chloro-2'-deoxyadenosine (cladribine), 6-methoxyguanine arabinoside (nelarabine), 6-thio-2'-deoxyguanosine, kinetin riboside, 2-chloro-2'-fluoroadenine arabinoside (clofarabine) were synthesized at the laboratory of molecular biotechnology, Institute of Microbiology and at the laboratory of chemistry of nucleotides and polynucleotides, Institute of Bioorganic Chemistry, National Academy of Sciences of Belarus.

Highly purified soybean lecithin Lipoid S100 (Lipoid GmbH, Germany) served as the source of phosphatidylcholine. The other reagents and solvents (chloroform, methanol, acetone) were of chemical and analytical purity grade or were additionally subjected to distillation.

Strain *S. netropsis* BIM B-428D provided from Belarussian collection of non-pathogenic microorganisms was cultured on the shaker (180 rpm) at 30 ^{0}C during 24 h in 250 ml Erlenmeyer flasks containing 100 ml of nutrient medium of the following composition (%): glucose – 1.0, yeast extract – 2.0, $MgSO_4 \times 7H_2O$ – 0.1; (initial pH 7.2). 18 h culture in amount of 10% (vol/vol) ratio was fed as the inoculum.

Upon separation of biomass by filtration PLD enzyme was precipitated from cultural filtrate with acetone (30–60% of final volume) for 15–20 min at 4 ^{0}C. The sediment was collected by centrifuging (5 000 g, 5 min) and dried under vacuum at ambient temperature.
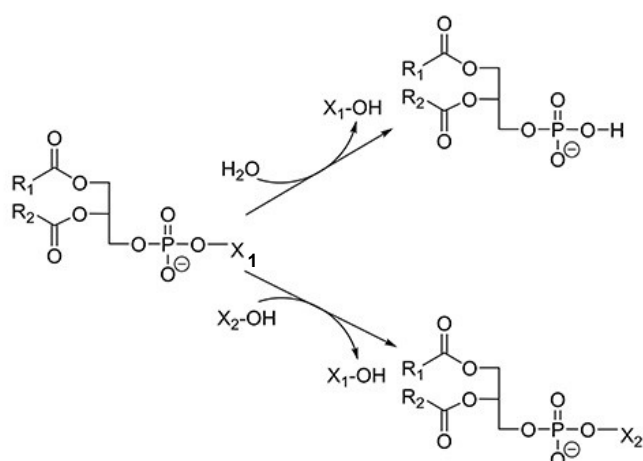
Fig. 1. Reactions of hydrolysis (upper) and transphosphatidylation (lower) catalyzed by PLD of *S. netropsis,* R_1 and R_2 – alkyls of fatty acid residues; X_1 and X_2 – polar groups

Analytical syntheses of phosphatidyl derivatives of nucleosides were carried out in 2 ml reaction mixture comprising 0.66 ml of 0.2 M sodium acetate buffer (pH 6.0), 0.1 M $CaCl_2$, 0.3 mg of biocatalyst (dry PLD enzyme preparation), 10 μmoles of nucleoside, 30 μmoles of phosphatidylcholine, 1.34 ml of chloroform.

All reactions were conducted at temperature 37 ^{0}C with constant vigorous stirring and were controlled by TLC on Silica gel 60 F254 plates (Merck, Germany) in the solvent systems chloroform/methanol/25% ammonia (or water) in the volumetric ratios 140:4:1.5 and 75:25:5. The compounds were detected in UV-light and eluted with distilled water (nucleosides) or ethanol (phospholipid derivatives).

Concentration of eluted substances were measured spectrophotometrically employing the known coefficient of molar extinction for specific nucleosides. It was taken into account that modification in the carbohydrate moiety of nucleoside molecule actually did not affect the values of molar extinction coefficient. The absorbance spectra of eluates were recorded at spectrophotometer PB2201A (Solare, Belarus).

Transphosphatidylation activity of PLD was expressed as nanomoles of the product formed in the course of enzymatic reaction performed during 1 min at 37 °C, pH 6.0 and calculated per 1 mg of enzyme preparation.

Preparative synthesis of phospholipid derivative of 2-chloro-2'-fluoroadenine arabinoside (clofarabine) occurred in biphasic reaction mixture of 10 ml volume consisting of 4 ml of 0.2 M sodium acetate buffer (pH 6.0) and 6 ml of chloroform. The mixture also contained 30 mg of nucleoside, 100 mg of phosphatidylcholine, 0.1 M $CaCl_2$ and 1.2 mg of PLD preparation.

After termination of the reaction chloroform layer was separated and evaporated. Phosphatidylclofarabine was recovered on Silica L60 (Merck, Germany) gel column by eluting

phospholipid with isocratic system of solvents – chloroform/methanol/water (75:25:5, vol/vol). Chromatographic peaks were revealed using UV-detector monitoring absorbance at 206 nm and 264 nm. The fractions containing chromatographically pure target compound (TLC control) were pooled and concentrated with the aid of rotor evaporation unit at 30 °C.

^{31}P, ^{13}C and ^{1}H NMR spectra were registered by Avance 500 device (Bruker, Germany). Chemical shifts are presented relative to tetramethylsilane as the internal standard.

3. Results and Discussion

Feasibility of producing phospholipid derivatives of several modified pharmacologically significant nucleosides was evaluated. It is clear from the data summed up in **Table 1** that all tested nucleosides were phosphatidylated by PLD with yields 44–95 mol% by 1–8 h of the reaction process.

Fig. 2 represents synthesis of phosphatidylclofarabine as the typical curve reflecting the dynamics of end product accumulation during the conducted enzymatic reactions. It is evident that the initial nucleoside was rapidly transformed into phospholipid derivative and further on underwent no changes (e.g. hydrolysis).

Noteworthy, that majority of the phosphatidyl derivatives of nucleosides shown in **Table 1** (brivudine, acyclovir, gancyclovir, et al.) appear to have been prepared by us for the first time. Derivatives of cladribine, fludarabine and clofarabine were also synthesized by Tsybulskaya et al. [14], applying laborious multistage chemical procedures.

Likewise the similar strategy was preferred for synthesis of phosphatidyl derivatives of other nucleosides [15, 30].

Preparative synthesis of liponucleotides was exemplified in this study by production of phosphatidylclofarabine. According to TLC data, product yield of the reaction reached 86 mol%. Following recovery of the target product and its purification on silica gel column, 65 mg of phosphatidylclofarabine was produced (making the final yield 68 mol% relative to amount of nucleoside supplied into reaction mixture).

Results of NMR spectroscopy of the synthesized conjugate agree well with those provided in the reports [14] for structurally similar compounds. Analyses of ^{1}H NMR spectrum revealed nucleoside signals 8.28 (c, H8), 7.90 (broad-range NH_2 signal), 6.36 (dd, $J_{F,H1}$ = 14.0 Hz; $J_{H1,H2}$ = 4.5 Hz; H1') and other signals partially overlapped by signals of lipid residues.
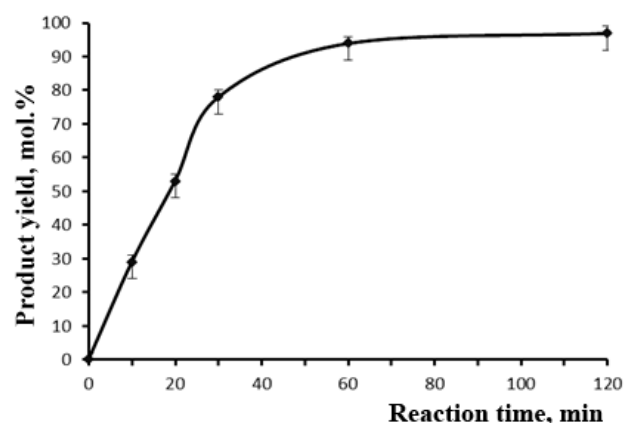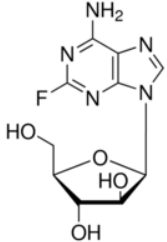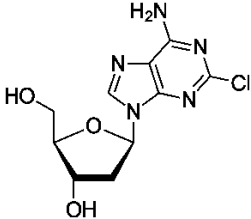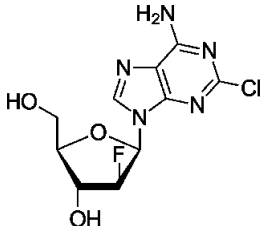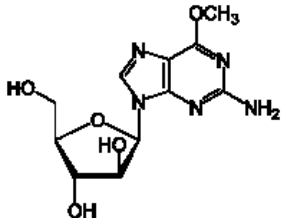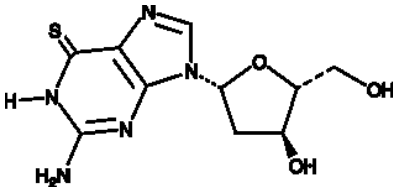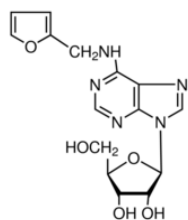

Fig. 2. Dynamics of phosphatidylclofarabine accumulation during transphosphatidylation reaction catalyzed by *S. netropsis* PLD
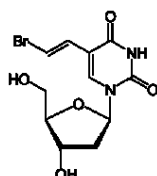
Table 1: Efficiency of liponucleotide synthesis

Nucleoside - acceptor	PLD activity (nmol/min·mg dry powder)	Max. conversion (%) to phosphatidyl-nucleosides	Time (h) for attaining max. conversion
2-Fluoroadenine arabinoside (Fludarabine)	67	44	5
2-Chloro-2'-deoxyadenosine (Cladribine)	952	>95	2–3
2-Chloro-2'-fluoroadenine arabinoside (Clofarabine)	1267	>95	1
6-Methoxyguanine arabinoside (Nelarabine)	567	82	4
6-Thio-2'-deoxyguanosine	617	70	3.5

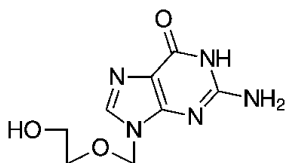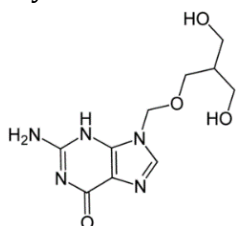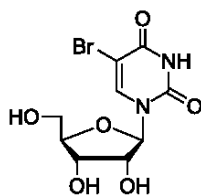| 6-Furfurylaminopurine riboside (Kinetin riboside) | 920 | >95 | 2 |
5-Bromovinyl-2'-deoxyuridine (Brivudine)	1560	>95	5
Acyclovir	243	75	5
Gancyclovir	903	85	8
5-Bromouridine (BrUrd)	880	61	2
5-Bromo-2'-deoxyuridine (BUdR)	1000	65	3
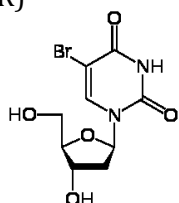
5-Iodo-2'-deoxyuridine (Idoxuridine)	1660	86	3
2-Chloro-2'-deoxyuridine (CldU)	1330	90	3
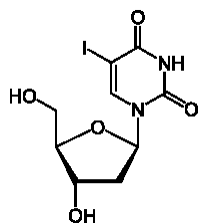
^{13}C NMR spectrum is represented by signals of two C=O groups at 172.92 and 172.71 md, nucleoside fragment, base – 157.23, 153.79, 150.62, 140.39 (d, $J_{C2',F}$ = 2.6 Hz; C8), 117.75 (C5), carbohydrate fragment – 95.42 (d, $J_{C2',F}$ = 192.4 Hz; C2'). Wide-range signal detected in ^{31}P spectrum at -0.68 md indicates unambiguously the presence of conjugate.
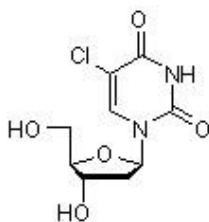
4. Conclusion

A mono-stage process conjugating several pharmacologically promising modified nucleosides with phospholipid was accomplished in the present study. Some liponucleotides were derived for the first time. Soybean lecithin acted as the donor of phosphatidyl residue.

PLD isolated from the previously selected strain *S. netropsis* BIM B-428D served as biocatalyst in transphosphatidylation reaction. The yield of liponucleotide synthesis reactions ranged from 44 to 95 mol% depending on the acceptor of phosphatidyl residue. Exemplificative preparative synthesis of 5'-phosphatidyl-2-chloro-2'-fluoroadenine arabinoside (phosphatidylclofarabine) has enabled to produce 65 mg (about 68 μmoles) of chromatographically pure phospholipid with the final yield 68 mol% calculated from the amount of modified nucleotide added into reaction mixture.

In general, the proposed enzymatic method of liponucleotide biosynthesis exceeds in some vital parameters widely practiced chemical processes, hence it may lay the basis for elaboration of efficient industrial technologies.

Conflict of interest

The authors clearly declared that they have no any conflict of interest.

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