Phospholipid derivatives of cladribine and fludarabine: Synthesis and biological properties

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Abstract
Phospholipid derivatives of anticancer nucleosides cladribine and fludarabine (F-ara-A) bearing 1,2- and 1,3-diacylglycerol moieties have been prepared by the H-phosphonate approach using 1,1,3,3-tetraisopropyldisiloxane-1,3-diyl protecting group for cladribine and a combination of tert-butyldimethylsilyl and levulinyl protecting groups for 2-fluoroadenine nucleosides. The synthesized conjugates exhibited lower in vitro antiproliferative activity against human tumor cell lines in comparison with the same concentrations of the parent cladribine and fludarabine phosphate. In the course of biokinetic study, it was found that intragastric administration of phospholipid F-ara-A derivatives to Wistar rats and ICR outbred male mice led to a slow release of F-ara-A into the bloodstream, a smooth increase in nucleoside concentration, and prolonged serum circulation of liberated nucleoside. The oral bioavailability of F-ara-A from 1,2-dimyristoylglycerophosphate derivative was similar to its oral bioavailability from fludarabine phosphate.

1. Introduction
Chemically modified purine nucleosides constitute an important class of antimetabolites widely used in the therapy of cancer. Most nucleoside anticancer agents are especially effective against hematological malignances. Among them, 2-chloro-2'-deoxyadenosine (cladribine, CdA) and 9-β-D-arabinofuranosyl-2-fluoroadenine 5'-monophosphate (fludarabine phosphate, F-ara-AMP) are extensively used in single and combinational regimen for the treatment of patients with chronic lymphocytic leukemia. These drugs are also effective against other malignancies such as hairy-cell leukemia, non-Hodgkin’s lymphomas, Waldenström’s macroglobulinaemia, and cutaneous T-cell lymphomas.

The usage of many nucleoside analogues is associated with a number of drawbacks such as poor pharmacokinetic properties, quick conversion into inactive metabolites in blood, low oral bioavailability, insufficient specificity and broad organ distribution leading to severe toxic effects. The use of CdA and F-ara-AMP is also connected with some limitations. Fludarabine phosphate is characterized by short plasma half-life and rapid metabolism, which are accompanied by several therapy-limiting side-effects. The oral bioavailability of halogenated analogue of F-ara-A, CdA, is moderate due to instability of cladribine in the acidic medium of stomach. Besides, some cells are resistant to F-ara-A and CdA because of decreased phosphorylation of these drugs by deoxycytidine kinase.

To enhance the efficiency of chemotherapy and diminish its toxicity, several drug delivery approaches have been proposed, among them the prodrug strategy. It is known that the phospholipids are hydrolyzed in the intestine by phospholipase A2 (PLA2) to produce the lyso-compounds, which, after absorption by the enterocytes, are reacylated and incorporated into chylomicrons with other lipoproteins. Chylomicrons are secreted into the mesenteric lymph and then flux throughout the thoracic lymph duct into the systemic circulation. It had been shown that the phospholipid prodrug approach can enhance the enterocyte absorption and promote lymphatic transport, thereby protecting the drugs from hepatic first-pass metabolism. The antitumor drugs can acquire modified pharmacokinetic and pharmacological properties when absorbed throughout the lymphatic system and help to avoid resistance mechanisms.

Since the 1970s, numerous investigations were devoted to the preparation of lipid prodrugs of nucleoside therapeutics and evaluation of their antiproliferative properties. A number of conjugates of 1-β-D-arabinofuranosylcytosine (ara-C) with natural and...
synthetic phospholipids, including 1-β-D-arabinofuranosylcytosine 5′-diphosphate-1,2-diacylglycerols and oxy- and thioether phospholipid derivatives, were shown to possess significant antitumor activity against various types of leukemia.17–18 Wide attention has been paid to the preparation of various lipid conjugates of gemicitabine including 5′-O-elicid, 5′-O-succinyl-cardiolipin, and 4-(N)-trisnorsqualenyl derivatives. The later compound, when administered intravenously or orally in the form of supramolecular nanoassemblies dispersed in aqueous medium, exhibited impressive in vivo antiproliferative activity along with improved pharmacokinetics and site-specificity.19,20 The ability of gemicitabine conjugate with 1-S-dodecyl-2-O-decylthiglycerol-3-phosphothiolic acid to overcome the cellular resistance to the parent nucleoside was demonstrated in vitro.21

We have recently described the synthesis of phospholipid derivatives of 1-(β-D-ribofuranosyl)-1H-1,2,4-triazole-3-carboxamide (ribavirin) and 2-chloro-9-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)adenine (clofarabine, CI-F-ara-A) containing the residue of natural 1,2-diacylglycerols and their synthetic 1,3-isomers.22,23 The antiproliferative activity of some clofarabine conjugates with 1,2- and 1,3-diacylglycerophosphates against HeLa (cervix carcinoma) and MCF-7 (breast carcinoma) cell lines was similar to the order of magnitude as that of parent clofarabine.22 We have also demonstrated that ribavirin conjugates with 1,3-diacylglycerophosphates can be hydrolyzed by porcine pancreatic lipase A2 (PLA2) although this reaction proceeds essentially slower in comparison with the hydrolysis of 1,2-diacyl derivatives.23 Similar phenomenon was previously observed for isomeric 1,2- and 1,3-diacylglycerophosphocholines.24–26 It was shown that PLA2 was able to hydrolyze not only natural 1,2-diacylglycerophosphocholines but their synthetic 1,3-diacyl regioisomers, in the later case the reaction occurred at C-1 atom of glycerol backbone with the release of corresponding fatty acid.24 The ability of 1,3-diacylglycerophosphate nucleoside derivatives to undergo enzymatic hydrolysis by PLA2 testifies that these compounds may, in principle, enter the first stage of metabolic activation analogously to their 1,2-counterparts. Since the rates of this reaction are different for 1,2- and 1,3-diacylglycerophosphate–nucleoside conjugates, we assumed that such isomeric compounds may possess different stability in the gastrointestinal tract as well as different pharmacokinetic properties.

In the literature, there are few data on F-ara-AMP prodrugs and, to our best knowledge, no information on the prodrugs of CdA. It is well known that F-ara-AMP itself is active against various leukemia cell lines but lacks activity against most solid tumors. Nevertheless, several alkylphospholipid and O-alkylglycerophospholipid derivatives of F-ara-A were reported to exhibit substantial efficiency against breast tumor cell lines (MaTu, MCF-7)27 that is alternative biological activity in comparison with the parent drug. The present work is aimed at the preparation and biological evaluation of phospholipid prodrugs of CdA and F-ara-AMP bearing 1,2- and 1,3-diacylglycerol moieties.

2. Results and discussion

2.1. Synthesis

The preparation of 1,2- and 1,3-diacylglycerophosphate derivatives of CdA and F-ara-A has been performed by the hydrogenophosphonate method used for the synthesis of phospholipids,27 lipid–nucleoside conjugates,28,29 and oligonucleotides.30 To prepare CdA derivative with free 5′-OH group suitable for the condensation with 1,2- and 1,3-diacylglycerol H-phosphonates, we exploited 1,1,3,3-tetraisopropylidiloxane-1,3-diy1 (TIPDS) protecting group widely used in nucleoside chemistry for blocking the hydroxyl functions of carbohydrate moiety.31–33 The treatment of cladribine (1) with 1,3-dichloro-1,1,3,3-tetraisopropylidiloxane (TIPDSCl2) (1.2 mol equiv) in pyridine led to the formation of cyclic 3′,5′-O-TIPDS cladribine derivative 2 (83%) (Fig. 1). The selective deblocking of 5′-hydroxy function of cyclic 3′,5′-O-TIPDS derivative 2 under the treatment with a mixture of trifluoroacetic acid/water/THF (1:1:4, v/v)34 for 2.5 h at 0°C led to the preparation of 3′-O-silylated nucleoside 3 isolated by column chromatography in 73% yield, along with isomeric 5′-O-silylated derivative 4 (22%).

The structure of isomeric nucleosides 3 and 4 was confirmed by NMR spectroscopy data. The peculiarities of 1H NMR spectra of these compounds were very similar to those we observed for the corresponding clofarabine derivatives.22 In the spectrum of 3′-O-silylated compound 3, the signal from H-3′ proton is markedly shifted to the low field in comparison with that of nucleoside 4 bearing free 3′-OH group (the corresponding multiplet is located at 4.73 ppm for compound 3 and at 4.44 ppm in the case of isomeric nucleoside 4). Correspondingly, a significant low-field shift of H-5′, H-5″ resonance is observed in the spectrum of 5′-O-silylated derivative 4 (the signals of these protons are located at 3.92–3.88 and 3.82 ppm, whereas in the case of compound 3 the corresponding multiplets are observed at 3.63 and 3.55 ppm). The presence of 5′-hydroxyl function in the molecule of nucleoside 3 is reflected in the appearance of the triplet from the proton of 5′-OH group at 5.09 ppm, while in the case of compound 4 the signal attributed to the proton of 3′-OH group is observed as a doublet at 5.36 ppm.

For the preparation of selectively protected F-ara-A derivative bearing free 5′-OH function we used a combination of tert-butylidimethylsilyl (TBDM) and levulinyl (Lv) protecting groups previously employed in the synthesis of 1-β-D-arabinofuranosyltosine (ara-C, cytarabine) and 9-β-D-arabinofuranosyladenine (ara-A, vidarabine) derivatives.17,35,36 Reaction of F-ara-A (21) with tert-butylidimethylsilyl chloride (TBDMCl) (1.8 mol equiv) in DMF in the presence of imidazole furnished 5′-O-silylated nucleoside 22 in 82% preparative yield. The acylation of 5′-O-TBDM-F-ara-A (21) with levulinic anhydride generated in situ from levulinic acid with DCC in the presence of 4-(dimethylamino)pyridine in EtOAc gave 2-fluoro-9′-[(tert-butylidimethylsilyl)-2,3- di-o-leuvinyl-1-β-D-arabinofuranosyl]adenine (23) in an excellent yield (99%). Analogously to previous observations for ara-A derivatives,35,36 we did not detect the formation of N-levulinated 2-F-adenine nucleosides in these conditions.

It was previously shown that the treatment of 5′-O-TBDMs-F-ara-A (21) with levulinic anhydride generated in situ from levulinic acid with DCC and with for the formation of 5′-OH-functionalized nucleoside 22 was confirmed by 1H NMR spectroscopy. The cytosine proton of ara-C, cytarabine) and 9-β-D-arabinofuranosyladenine (ara-A. Reacting F-ara-A (21) with tert-butylidimethylsilyl chloride, the protection of 5′-OH group is observed as a doublet at 5.36 ppm.

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presence of pivaloyl chloride (3 mol equiv) in pyridine. After oxidation with the solution of I₂ in CH₂Cl₂/Py/H₂O (3:1:1, v/v/v) mixture, phosphodiester 13–16 and 25–28 were isolated by silica gel chromatography. To remove the protective groups from the obtained conjugates, 1 M tetrabutylammonium fluoride in THF for 5′-O-TIPDS group and hydrazine hydrate in pyridine containing 20% AcOH for levulinyl groups were used. The deprotected lipid–nucleoside conjugates were purified by a silica gel column chromatography and isolated after the treatment with 1 M NaI in acetone or dry CH₃CN as sodium salts 17–20 and 29–32.

Conjugates 17–20 were obtained in 68–81% overall yields (calculated on nucleoside 3) and compounds 29–32—in 70–88% overall yields (calculated on nucleoside 24).

The structure of the synthesized phospholipid–nucleoside conjugates are confirmed by the data of NMR spectroscopy including 2D techniques. The registered ¹H and ¹³C NMR spectra of the synthesized derivatives contain the signals from all H- and C-atoms of every structural fragment (carbohydrate, heterocyclic, glycerol, and acyl residues). In the ³¹P NMR spectra of compounds 17–20 and 29–32, the signals from P-atom are observed in the region characteristic for previously synthesized phospholipid derivatives of ribavirin and clofarabine.

The ¹⁹F NMR spectra of fludarabine conjugates 29–32 exhibit the characteristic peaks from F-atom near ~51 ppm. The presence of fluorine atom in the heterocyclic base of compounds 29–32 leads to the splitting of the signals from C(2), C(6), C(4), and C(5)
atoms into doublets (coupling constants $J_{C(2)F} \approx 203$ Hz, $J_{C(1)F} \approx 20$ Hz, $J_{C(4)F} \approx 20$ Hz, $J_{C(5)F} \approx 4$ Hz).

The differences in the structure of isomeric 1,2- and 1,3-diacylglycerophosphate nucleoside derivatives are reflected in the features of their $^{13}$C NMR spectra similar to those previously described for the corresponding phospholipid–ribavirin conjugates. The two-bond C, P coupling attributed to (C(3)) atom of glycerol moiety attached to the phosphate group is observed in $^{13}$C NMR spectra of 1,2-diacyl isomers 17, 18, and 29, 30, with $J_{C(2)P}$ equal to 3.5–4.0 Hz, whereas in the case of 1,3-diacyl derivatives 19, 20 and 31, 32 such coupling is observed for C(2) atom resonance, with $J_{C(2)P}$ in the range 4.0–5.0 Hz. Furthermore, in the $^{13}$C NMR spectra of 1,2-diacylglycerophosphate derivatives of cladribine and fludarabine the three-bond coupling between phosphorus and C(2) atom of glycerol fragment is observed ($J_{C(2)P} = 7.0–7.5$ Hz), while the spectra of 1,3-diacyl isomers are characterized by the presence of the three-bond C,P couplings involving C(1) and C(3) atoms of glycerol residue ($J_{C(1)P} = 3.5–4.5$ Hz).

The data of mass-spectrometry are in a good accordance with the composition of the synthesized compounds.

### 2.2. The antiproliferative activity of diacylglycerophosphate nucleoside derivatives

In vitro antiproliferative activity of cladribine derivatives 17–20 (in 0.1–100 μM concentration) against human hematologic and solid tumor cell lines (acute myelogenous leukemia KG-1, chronic myelogenous leukemia K-562, acute T lymphoblastic leukemia MOLT-3, and myeloma RPMI-8226; liver adenocarcinoma SK-HEP-1, breast adenocarcinoma MCF-7, breast carcinoma ZR-75-1, Burkitt’s lymphoma Raji, breast adenocarcinoma MCF-7, breast carcinoma ZR-75-1, uterus leiomyosarcoma SK-UT-1B) was evaluated in comparison with parent nucleoside cladribine (1). The obtained results are given in Table 1.

It was found that 1,2-dimyristoylglycerophosphate cladribine derivative (17) inhibited the growth of several hematologic tumor cell lines (MOLT-3, KG-1, and K-562 leukemia) and did not inhibit solid tumor cell cultures. Compounds 18–20 did not inhibit both hematologic and solid tumor cells.

Cladribine (1) and compound 17 inhibited MOLT-3 cells at micromolar concentrations ($IC_{50}$ value was equal to 2.3 μM for nucleoside 1 and 8.0 μM for conjugate 17). The concentration–effect curves of cladribine (1) and conjugate 17 for this cell line come together at maximal concentration used (10–100 μM, Fig. 2).

Acute myelogenous leukemia KG-1 cells are very sensitive to cladribine; in this cell line, $IC_{50}$ value of nucleoside 1 was found to be equal to 0.2 μM, whereas the corresponding value of its conjugate 17 was higher by about two orders of magnitude. In the experimental conditions, chronic myelogenous leukemia K-562 cells turned to be moderately sensitive to both nucleoside 1 and conjugate 17 which were characterized by $IC_{50}$ values equal to 10 and 50 μM correspondingly.

In vitro antiproliferative activity of fludarabine derivatives 29–32 (in 0.1–100 μM concentration) against promyelocytic leukemia HL-60, acute myelogenous leukemia KG-1, chronic myelogenous leukemia K-562, acute T lymphoblastic leukemia MOLT-3, Burkitt’s lymphoma Raji, breast adenocarcinoma MCF-7, breast carcinoma ZR-75-1, uterus leiomyosarcoma SK-UT-1B, and cervix carcinoma M-HeLa clone 11 was evaluated in comparison with F-ara-AMP. The obtained results are presented in Table 2.

#### Table 1

In vitro antiproliferative activity of cladribine (1) and its phospholipid derivatives 17–20, $IC_{50}$ (μM)

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Compound</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>MOLT-3</td>
<td>23.6 ± 0.7</td>
</tr>
<tr>
<td>KG-1</td>
<td>0.2 ± 0.04</td>
</tr>
<tr>
<td>K-562</td>
<td>10 ± 2.02</td>
</tr>
<tr>
<td>RPMI-8226</td>
<td>6.2 ± 2.47</td>
</tr>
<tr>
<td>SK-HEP-1</td>
<td>4 ± 0.91</td>
</tr>
<tr>
<td>MCF-7</td>
<td>45 ± 14.57</td>
</tr>
<tr>
<td>ZR-75-1</td>
<td>&gt;100</td>
</tr>
<tr>
<td>SK-UT-1B</td>
<td>1 ± 0.53</td>
</tr>
</tbody>
</table>

ND = not determined.

<sup>a</sup> $IC_{50}$ is the concentration of the compound that inhibits cell growth by 50% compared with untreated control. $IC_{50}$ values are given as means ± SD (standard deviation) of three independent experiments done in triplicate. $P<0.05$ was considered significant.

<sup>b</sup> $P<0.01$.

<sup>c</sup> $P<0.001$.

<sup>d</sup> $P<0.0001$.

#### Table 2

In vitro antiproliferative activity of F-ara-AMF and fludarabine conjugates, $IC_{50}$ (μM), 48 h (72 h)

<table>
<thead>
<tr>
<th>Cell line</th>
<th>F-ara-AMP</th>
<th>Compound</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>29</td>
<td>30</td>
</tr>
<tr>
<td>HL-60</td>
<td>0.09 ± 0.02</td>
<td>30 ± 3.61&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>KG-1</td>
<td>0.15 ± 0.03</td>
<td>55 ± 15.00&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>K-562</td>
<td>0.40 ± 0.05</td>
<td>45 ± 8.66&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>MOLT-3</td>
<td>0.95 ± 0.38</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Raji</td>
<td>0.40 ± 0.09</td>
<td>&gt;100</td>
</tr>
<tr>
<td>MCF-7</td>
<td>0.13 ± 0.02</td>
<td>45 ± 13.23&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>ZR-75-1</td>
<td>0.6 ± 0.1</td>
<td>&gt;100</td>
</tr>
<tr>
<td>SK-UT-1B</td>
<td>6.0 ± 0.87</td>
<td>&gt;100</td>
</tr>
<tr>
<td>M-HeLa cl. 11</td>
<td>2.0 ± 0.87</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

ND = not determined.

<sup>a</sup> $IC_{50}$ is the concentration of the compound that inhibits cell growth by 50% compared with untreated control. $IC_{50}$ values are given as means ± SD (standard deviation) of three independent experiments done in triplicate. $P<0.05$ was considered significant.

<sup>b</sup> $P<0.001$.

<sup>c</sup> $P<0.01$.

<sup>d</sup> $P<0.002$.

<sup>e</sup> $P<0.03$.

<sup>f</sup> $P<0.01$.

<sup>g</sup> $P<0.05$. 

![Figure 2](image-url)
1,2-Dimyristoylglycerophosphate-fludarabine conjugate 29 inhibited the growth of leukemia HL-60, KG-1, K-562 cells as well as breast carcinoma MCF-7 cell line, although the values of IC_{50} for this compound were more than two orders of magnitude higher in comparison with F-ara-AMP. We observed the effect of prolonged exposure (72 h versus 48 h) on the viability of HL-60, KG-1, and MCF-7 tumor cell cultures upon the treatment with compound 29. Similar effect was also observed in the case of 1,3-dimyristoyl derivative 31. Probably, this phenomenon is due to a slow release of the active drug moiety from the lipid derivatives in vitro.

The obtained results are in accordance with the known data on decreased in vitro activity of lipid nucleoside derivatives in comparison with unmodified nucleosides, whereas the results of the in vivo testing often demonstrated improved biokinetic properties, along with equal or even enhanced activity of such derivatives versus their parent nucleosides.17,40-42

2.3. Pharmacokinetic properties

To estimate the pharmacokinetic properties of fludarabine conjugates with isomeric 1,2- and 1,3-diacylglycerophosphate, F-ara-AMP (30 mg/kg, 0.08 mmol/kg) and conjugates 29–32 (29, 31 in a dose 72 mg/kg, 30, 32–77 mg/kg, equimolar to 30 mg/kg of F-ara-AMP) were intragastrically administered as single doses to Wistar rats. It is well known that, after F-ara-AMP administration, a rapid and quantitative hydrolysis of the nucleotide to F-ara-A, principal metabolite, is observed in plasma, serum, and tissues; then F-ara-A is taken up by cells and rephosphorylated by cellular deoxycytidyl kinase to the cytoxic triphosphate F-ara-ATP. Thus, in this study, the main attention was given to the determination of F-ara-A concentration in serum of animals after administration of lipid fludarabine derivatives in comparison with unmodified F-ara-AMP. The concentration of F-ara-A in serum samples was determined by HPLC, and time dependences of F-ara-A concentration were plotted for each of the investigated compounds.

In the course of this preliminary pharmacokinetic study, it was found that intragastric administration of phospholipid fludarabine derivatives 29–32 to Wistar rats led to slower release of fludarabine into the systemic bloodstream, smooth increase of nucleoside concentration in serum, and prolonged elimination as compared with analogous administration of fludarabine phosphate. The bioavailability of F-ara-A (21) from compounds 29–32 was moderate to high versus its bioavailability from F-ara-AMP. The area under the curve from zero to 24 h (AUC_{0-24}) for F-ara-A released from conjugate 29 was very close to that observed in the case of unmodified fludarabine phosphate (~17,000 ng/h/mL).

Based on the results of the above described in vitro and in vivo experiments, we have chosen compound 29 for more detailed investigation of its pharmacokinetic parameters. Fludarabine phosphate (50 mg/kg; 0.137 mmol/kg) and conjugate 29 in an equimolar dose 120.4 mg/kg were intragastrically administered as single doses to ICR outbred male mice. The serum samples were obtained from the blood collected in 0.5, 1, 2, 4, 8, and 24 h after administration of the investigated compound and the concentration of F-ara-A was determined by HPLC. The time dependences of fludarabine concentration in the serum of mice after administration of F-ara-AMP and compound 29 are given in Figure 3.

When phospholipid fludarabine derivative 29 was given to the mice, the maximal F-ara-A concentration (C_{max}) 1613 ± 247 ng/mL was observed in mice serum, versus 8989 ± 2537 ng/mL observed in the case of F-ara-AMP administration. For conjugate 29, the time to maximal F-ara-A concentration (T_{max}) was equal to 2.8 ± 1.1 h and F-ara-A apparent half-life (T_{1/2})—to 14.2 ± 2.6 h (compare with 0.9 ± 0.2 h and 1.5 ± 0.4 h, correspondingly, that observed after administration of F-ara-AMP).

The serum concentration of F-ara-A in 24 h after administration (C_{min}) was markedly higher in the case of intragastrically administered conjugate 29 than in the case of unmodified fludarabine phosphate (199 ± 31 and 13 ± 9 ng/mL, correspondingly) (Fig. 3).

Thus, when lipid fludarabine derivative 29 was intragastrically administered to mice, C_{max} of F-ara-A in animal serum was 5.6 times lower than after administration of fludarabine phosphate. Besides, compound 29 was characterized by three-fold increase in T_{max} and by essentially decreased elimination rate of F-ara-A. Hence phospholipid conjugate 29 behaves as fludarabine prodrug. The differences between the mean values of pharmacokinetic parameters of compound 29 and F-ara-AMP were statistically significant (P < 0.001 for C_{max}, C_{min}, and T_{1/2}; P < 0.01 for T_{max}).

The areas under the curves from zero to 24 h (AUC_{0-24}) of F-ara-A released from conjugate 29 was equal to 19,368 ± 1411 ng·h/mL that was very close to the corresponding values obtained in the case of unmodified fludarabine phosphate administration (17,790 ± 1934 ng·h/mL; P < 0.25). Thus, the oral bioavailability of fludarabine from its phospholipid prodrug 29 is close to its oral bioavailability from the equimolar F-ara-AMP.

3. Conclusions

A series of new conjugates of antitumor nucleosides cladribine and fludarabine with natural 1,2-diacylglycerophosphates and their synthetic 1,3-counterparts was synthesized by the H-phosphonate method with the use of 1,1,3,3-tetraisopropylsiloxane-1,3-diy1 protection for cladribine derivatives and a combination of tert-butylidimethylsilyl and levulinic protecting groups for the preparation of fludarabine compounds.

Investigation of the antiproliferative activity of the obtained conjugates against a number of human leukemic and solid tumor cell lines has shown that the tested compounds exhibited low in vitro activity versus parent cladribine or fludarabine phosphate at the same concentrations. The results of preliminary pharmacokinetic study of diacylglycerophosphate fludarabine derivatives in animals testify that the synthesized compounds behave as fludarabine prodrugs and are characterized by a slow fludarabine release into the bloodstream and prolonged circulation of nucleoside in
animal serum. Further investigation of tissue distribution and in vivo antiproliferative properties of the synthesized lipid–nucleoside conjugates are necessary to estimate the potential of these compounds as antitumor agents.

4. Experimental

4.1. Chemistry

TLC was conducted on silica gel sheets Merck 60 F 254. Lipid derivatives were visualized on TLC by spraying with 30% H2SO4 and charring, and by the formation of phosphomolybdenum blue. Preparative column chromatography was performed on Sepacore® Flash Silica Cartridges (40–63 μm) (Buchi). NMR spectra were registered on an Avance 500 spectrometer (Bruker-Biospin). In 1H and 13C NMR spectra, chemical shifts (δ) are given in ppm relative to SiMe4 coupling constants (J) in Hz. In 13C NMR spectra, chemical shifts are given in ppm relative to external 2HPO4. Assignment of the signals in 1H and 13C NMR spectra was carried out with the use of 1H–1H and 13C–13C correlation spectroscopy. The solvents were purified and dried according to standard procedures before usage in the synthesis. High-resolution mass-spectra (HRMS) were recorded on Agilent 1290-6550 Q-TOF liquid chromatography mass-spectrometer (USA) using ESI (electrospray ionization).

1,2-Dipalmitoyl- and 1,2-dimyristoyl-sn-glycerol (5, 6) were prepared by the known methods starting from o-mannitol17,26, 1,3-dipalmitoyl- and 1,3-dimyristoylglycerol (7, 8) were obtained by the thermal isomerization of its 1,2-isomers.27

4.1.1. Synthesis of protected nucleosides

4.1.1.1. 2-Chloro-9-[2-deoxy-3-0-(1,1,3,3-tetraisopropylsiloxane-3-yl)-β-D-furanosyl]adenine (1). 1H NMR (DMSO-d6): δ 8.37 (1H, H-8); 7.84 (br s, 2H, NH2), 6.27 (dd, 1H, J = 8.0, J = 6.0, H-1'), 6.14 (1H, J = 3.5, J = 12.5, H-5'), 3.94 (m, 1H, H-4'), 3.63 (m, 1H, H-5'), 3.55 (m, 1H, H-5'), 2.77 (dd, 1H, J = 8.0, J = 5.5, J = 13.0, H-2'), 2.35 (dd, 1H, J = 8.0, J = 3.0, J = 13.0, H-2'), 2.17, 19.04, 17.01, 13.00, 12.39, 12.35 (i-Pr). HRMS (ESI): m/z, calcd for C52H60N3O2Si2[+M+H]+ 754.2335, found: 754.2330.

4.1.1.2. 2-Chloro-9-[2-deoxy-3-O-(1-hydroxy-1,1,3,3-tetraisopropylsiloxane-3-yl)-β-D-furanosyl]adenine (2). To the solution of caddriline 1H NMR (DMSO-d6): δ 8.22 (s, 1H, H-8), 7.82 (br s, 2H, NH2), 6.23 (dd, 1H, J = 8.0, J = 6.0, H-1'), 5.10 (dt, 1H, J = 7.0, J = 8.0, J = 5.5, J = 8.0, J = 3.5, J = 12.0, H-5'), 3.88 (dd, 1H, J = 12.5, J = 12.0, H-5'), 3.73 (m, 1H, H-4'), 2.84 (dd, 1H, J = 8.0, J = 13.0, H-2'), 2.56 (dd, 1H, J = 8.0, J = 13.0, H-2'), 1.30–0.90 (m, 28H, 4-i-Pr). 13C NMR (DMSO-d6): δ 156.63 (C(6)), 152.85 (C(2)), 150.56 (d, J = 13.0, C(6)), 149.23 (C(4)), 140.00 (C(8)), 138.66 (C(3)), 136.86 (C(3)), 125.68 (C(5)), 120.88 (C(5)), 62.52 (C(5)), 38.50 (C(5)), 21.72, 17.03, 169.66, 163.83, 163.66, 124.16, 123.34, 118.89, 118.82 (i-Pr). HRMS (ESI): m/z, calcd for C52H60N3O2Si2[M+H]+ 754.2229, found: 754.2224.

4.1.1.3. 2-Chloro-9-[2-deoxy-3-O-(1-hydroxy-1,1,3,3-tetraisopropylsiloxane-3-yl)-β-D-furanosyl]adenine (3). To the solution of nucleoside 2 (1.2 g, 2.27 mmol) in THF (24 mL), a mixture of trifluoroacetic acid/water (1:1, v/v) (12 mL) was added at 0 °C. After stirring for 2.5 h, the reaction mixture was poured into a cooled mixture of 5% NaHCO3 (100 mL) and EtOAc (350 mL). The organic layer was separated, washed with water (100 mL) and brine (100 mL), dried over anhydrous sodium sulfate, and evaporated. The residue was applied on a silica gel flash cartridge (40 g), and the products were eluted with a mixture of toluene/CH2Cl2/Et3N (45:45:10, v/v/v). The appropriate fractions were combined and evaporated to give nucleosides 3 (white powder, 0.96 g, 73%) and 4 (solid foam, 0.29 g, 22%).

Compound 3: 1H NMR (DMSO-d6): δ 8.37 (1H, H-8), 7.84 (br s, 2H, NH2), 6.27 (dd, 1H, J = 8.0, J = 6.0, H-1'), 6.14 (1H, J = 3.5, J = 12.5, H-5'), 3.94 (m, 1H, H-4'), 3.63 (m, 1H, H-5'), 3.55 (m, 1H, H-5'), 2.77 (dd, 1H, J = 8.0, J = 5.5, J = 13.0, H-2'), 2.35 (dd, 1H, J = 8.0, J = 3.0, J = 13.0, H-2'), 2.17, 19.04, 17.01, 13.00, 12.39, 12.35 (i-Pr). HRMS (ESI): m/z, calcd for C52H60N3O2Si2[+M+H]+ 754.2335, found: 754.2330.

4.1.1.4. 2-Fluoro-9-[2-deoxy-3-O-(1-hydroxy-1,1,3,3-tetraisopropylsiloxane-3-yl)-β-D-furanosyl]adenine (22). To the solution of fludarabine (21, 500 mg, 1.75 mmol), and imidazole (429 mg, 6.30 mmol) in DMF (1.5 mL), tert-butylidimethylsilyl chloride (474 mg, 3.15 mmol) was added under an argon atmosphere. After stirring for 16 h, the reaction mixture was evaporated, co-evaporated with toluene (5 mL), and poured into water (32 mL). The precipitate was filtered off, washed with water (15 mL), and crystallized from a mixture of water/MeOH/EtOH (20:10:7, v/v/v, 7 mL) to yield nucleoside 22 (575 mg, 82%).

4.1.1.5. 2-Fluoro-9-[5-O-(tert-butylidimethylsilyl)-β-D-arabinofuranosyl]adenine (23). To the solution of levulinic acid (961 mg, 8.28 mmol) in EtOAc (31 mL), DCC (202 mg, 1.65 mmol), and EtOAc (31 mL). After stirring for 40 min, EtOH (5 mL) was added, and the reaction mixture was stirred for additional 30 min. The precipitate was filtered off, the filtrate was diluted with EtOAc (200 mL) and extracted with cold 5% NaHCO3 (3 × 70 mL) and water (3 × 70 mL). The organic layer was dried over anhydrous sodium sulfate and evaporated. After purification by silica gel chromatography (25 g flash cartridge, gradient of
MeOH (0–25%) in CHCl₃), compound 23 (812 mg, 99%) was obtained as white powder.

¹³C NMR (CDCl₃): δ 8.15 (s, 1H, H-8), 6.44 (dd, 1H, J₁,₂ 4.5, H-1’), 6.19 (br s, 2H, NH₂), 5.57 (m, 2H, H₂-’), 4.09 (m, 1H, H₂-’), 4.00 (dd, 1H, J₃,₄ 3.5, J₅,₆ 11.5, H-5’), 3.93 (dd, 1H, J₃,₄ 4.0, J₅,₆ 11.5, H-5’), 2.88–2.21 (m, 8H, 2CH₂), 2.20 (s, 3H, CH₃), 2.08 (s, 3H, CH₃), 0.94 (s, 9H, (CH₃)₃Si), 0.13 (s, 3H, (CH₃)₂Si), 0.12 (s, 3H, (CH₃)₂Si). ³¹P NMR (CDCl₃): δ 202.65, 206.50 (2CH₂(CO)), 171.56, 170.92 (2OC(O)), 159.18 (d, J₆,C₂ 211.5, C(6)), 157.04 (d, J₆,C₂ 20.0, C(6)), 151.16 (d, J₆,C₂ 19.5, C(4)), 140.00 (C(8)), 117.36 (C(5)), 82.38, 81.84 (C(1’), C(4’)), 75.54, 74.52 (C(2’), C(3’)), 61.93 (C(3)), 37.85, 37.42, 29.75, 29.61, 27.80, 27.45 (2CH₂(CO)CH₂CH₂CO)), 25.93 ((CH₃)₂Si), 18.48 ((CH₃)₂Si), –5.40 ((CH₃)₂Si). ³¹F NMR (CDCl₃): δ –52.24. HRMS (ESI): m/z, calcd for C₃₀H₄₆N₅O₈Si [M+Na⁺] + 618.2369, found: 618.2369.

4.1.6. 2-Fluoro-9-[2,3-di-O-levulinyl-p-o-arabinofuranosyl]-adenine [24]. To the solution of nucleoside 23 (800 mg, 0.34 mmol) in pyridine (4 mL), 70% HF-pyridine (1.5 mL) was added at 5°C. After stirring for 2 h, the mixture was diluted with CHCl₃ (300 mL), washed with 5% NaHCO₃ (2 × 100 mL) and water (1 × 100 mL), dried over anhydrous sodium sulfate, evaporated, and co-evaporated with toluene (5 mL). The residue was applied at 5°C (2.5 mL) and the solution of Et₃N (0.12 (s, 3H, (CH₃)₃Si), 1.60 (m, 4H, 2CH₃), 0.88 (m, 6H, 2CH₃(C₂H₅)₂CO), 0.88 (s, 6H, 2CH₃(CH₂)]₃CO). ¹³C NMR (CDCl₃): δ 173.48 (CH₃(C₂H₅)₂CO), 69.86 (d, J₈,C₂ 4.5, H-2’), 63.77, 63.75 (H-1, H-3’ Gly), 45.58 ((CH₃(CH₂)₂N), 34.13, 31.94, 29.71, 29.68, 29.65, 29.51, 29.38, 29.31, 28.46, 22.70 (CH₃(CH₂)₂CO), 14.13 (CH₃(CH₂)₂N), 4.43 ((CH₃)₂Si). ³¹P NMR (CDCl₃): δ 4.62 (dd, 1J₈,P 61.10, 1J₈,H 11.0). HRMS (ESI): m/z, calcd for C₃₅H₄₅N₅O₈Si [M–Et₃N+Na⁺] + 655.4679, found: 655.4671.

4.1.3. General procedure for the preparation of conjugates 17–20

The mixture of nucleoside 3 (1 mmol) and H-phosphonate (9–12, 1.6 mmol) was evaporated with pyridine (3 mL), the residue was dissolved in pyridine (3 mL) and pivaloyl chloride (0.36 mL, 3 mmol) was added to the solution. After stirring for 4 min, the solution of I₂ (406 mg, 1.6 mmol) in CH₂Cl₂/py/water mixture (3:1:1, v/v/v; 5 mL) was added. The reaction mixture was kept for 1 h, then diluted with CHCl₃/Et₂N mixture (97:3, v/v; 300 mL) and washed with the solution of Na₂S₂O₃ (2 g) in brine (100 mL). The organic layer was dried over anhydrous sodium sulfate and evaporated; the residue was applied on silica gel flash cartridge (40 g), and the products were eluted with a gradient of MeOH (0–7%) in CHCl₃ containing 0.4% Et₂N. The appropriate fractions were combined and evaporated to give triethylammonium salts of phosphodiesters 13–16 as transparent oils.

Each of compounds 13–16 was dissolved in THF (20 mL), and 1M tetrabutylammonium fluoride in THF (3 mL) was added to the solution. After stirring for 1 h, the reaction mixture was evaporated and purified by silica gel chromatography (40 g flash cartridge; a gradient of MeOH (0–16%) in CHCl₃ contained 0.4% Et₃N). The appropriate fractions were combined and evaporated; the residue was dissolved in CHCl₃ (0.4 mL) and 1M NaI in acetone (1.6 mL) and CH₃CN (60 mL) were added to the solution. The precipitate was filtered off and washed with CH₃CN. After drying in vacuo over P₂O₅, the sodium salts of conjugates 17–20 were obtained as white powders in 68–81% yield calculated on nucleoside 3.

4.1.3.1. [2-Chloro-9-(2-deoxy-p-furanosyl)adenine]–5′-(12-di-O-myristoylsn-glycerol-3-yl)phosphate, sodium salt (17). ¹³C NMR (CDCl₃): δ 8.65 (s, 1H, H-8), 7.91 (br s, 2H, NH₂), 6.42 (t, 1H, J₃,₄ 6.5, J₁,₂ 6.5, H-1, H-1’), 5.19 (m, 1H, H-2’ Gly), 4.66 (m, 1H, H-3’), 4.42 (dd, 1H, J₁,₂ 12.0, J₂,₃ 3.0, H-1’ Gly), 4.20 (dd, 1H, J₁,₂ 12.0, J₂,₃ 7.0, H-1 Gly), 4.09 (m, 1H, H₄’), 3.99 (m, 2H, H₅’–H₇’), 3.94 (m, 4H, 2H₃-(CH₂)₂CH₂CO), 2.77 (m, 1H, H-2’ Gly), 2.42 (dd, 1H, J₆,₇ 15.0, J₆,₇ 3.5, J₅,₆ 13.0, H-2’), 2.31 (m, 4H, 2CH₃(CH₂)₂CH₂CO), 1.56 (m, 4H, 2CH₃(CH₂)₂CH₂CO), 1.28 (m, 40H, 2CH₃(CH₂)₂CH₂CO), 0.88 (t, 6H, 2CH₃(CH₂)₂CO). ³¹C NMR (CDCl₃): δ 173.49, 173.27 (2CH₂(CH₂)₂CO), 157.27, 154.05, 150.94, 141.00, 118.24 (Ada, 87.09 (d, J₄,C 8.5, C(4’)), 83.91 (C(1’)), 72.53 (C(1’)), 71.08 (d, J₆,C 8.0, C(2’)), 66.24 (d, J₆,C 5.0, C(5’)), 63.56 (d, J₆,C 3.5, C(3’)), 62.81 (C-1 Gly), 40.33 (C(2’)), 34.15, 33.59, 32.01, 29.73, 29.44, 29.13, 25.03, 22.73 (CH₃(CH₂)₂CO), 13.95 (CH₃(CH₂)₂CO). ³¹P NMR (CDCl₃): δ 0.11. HRMS (ESI): m/z, calcd for C₃₄H₄₂Cl₂N₂O₁₀ [M–Na₂H₂O] + 860.7405, found: 860.4697.

4.1.3.2. [2-Chloro-9-(2-deoxy-p-furanosyl)adenine]–5′-(12-di-O-palmitoylsn-glycerol-3-yl)phosphate, sodium salt (18). ¹³C NMR (CDCl₃): δ 8.63 (s, 1H, H-8), 7.92 (br s, 2H, NH₂), 6.40 (t, 1H, J₁,₂ 6.5, H-1’ Gly), 5.09 (m, 2H, H₂-’), 4.09 (m, 1H, H₂-’), 4.00 (dd, 1H, J₃,₄ 3.5, J₅,₆ 11.5, H-5’), 3.93 (dd, 1H, J₃,₄ 4.0, J₅,₆ 11.5, H-5’).
5.4. General procedure for the preparation of conjugates 29-32

The condensation of nucleoside 24 (1 mmol) and H-phosphonates 9-12 (1.6 mmol) was performed as described above for the preparation of phosphodiester 13-16.

Each of compounds 25-28 was isolated by column chromatography as transparent oil and dissolved in Py/ACOH mixture (4:1, v/v, 17.5 mL), then hydrizine hydrate (0.44 mL) was added to the solution. After stirring for 20 min, the reaction mixture was evaporated, and the residue was evaporated to dryness (3 mL). The obtained oil was applied on a silica gel flash cartridge (25 g), and the products eluted with a gradient of CH3OH (0-20%) in CHCl3 contained 0.4% Et3N. The appropriate fractions were combined and evaporated; the residue was dissolved in the mixture of CHCl3/CH3OH/water (4:6:1, v/v/v, 0.3 mL), and 1 M NaI in acetone (1.62 mL) and CH3CN (50 mL) were added. The precipitate was filtered off and washed with CH2CN to give, after drying in vacuum over P2O5, compounds 29-32 (sodium salts) as white powders in 70-88% yield calculated on nucleoside 24.

4.1.4.1. [2-Fluoro-9-(β-D-arabinofuranosyl)adenine]-5'-[(1,2-di-o-palmitoyl-sn-glycerol-3-y]phosphate, sodium salt (31). H NMR (DMSO-d6): δ 8.17 (s, 1H, H-8), 7.79 (br s, 2H, NH2), 6.08 (d, 1H, J1,2 = 5.0, H-1'), 5.87 (br s, 2H, 2-0H, 3-0H), 5.03 (m, 1H, H-2'), 4.27 (d, 1H, J1,1 = 12.0, J1,2 = 3.0, H-1 Gly), 4.16 (m, 1H, J1,1 = 12.0, J1,2 = 3.0, H-1 Gly), 4.12 (m, 1H, J1,1 = 12.0, J1,2 = 3.0, H-1 Gly), 3.88 (m, 2H, H-2', H-3'), 3.82 (m, 1H, H-4'), 3.71 (m, 2H, 2-0H Gly), 2.22 (m, 4H, 2CH2(CH2)3CH3CO), 1.22-1.21 (40H, 2CH3(CH2)2CH3CO), 0.84 (t, 6H, 2CH3(CH2)2CH3CO), 13C NMR (DMSO-d6): δ 172.51, 172.24 (2CH3(CH2)2CO), 158.52 (d, JCF3 = 203.0, C(2)), 157.34 (d, JCF3 = 20.5, C(6)), 150.59 (d, JCF3 = 19.5, C(4)), 140.62 (d, JCF3 = 4.5, C(5)), 83.43 (C(1')), 85.23 (d, JCF3 = 5.5, C(5')), 75.33, 74.85 (C(2'), C(3')), 70.43 (d, JCF3 = 5.0, C(5)), 63.80 (d, JCF3 = 5.0, C(5')), 62.40 (d, JCF3 = 4.0, 62.29 (C-1 Gly), 33.51, 33.33, 31.24, 28.99, 28.87, 28.67, 28.37, 24.74, 23.49, 23.44, 22.04 (CH2(CH3)2CO), 13.88 (CH3(CH2)2CO). 13P NMR (DMSO-d6): δ = -0.03. 31P NMR (DMSO-d6): δ = -50.89. HRMS (ESI): m/z, calcd for C30H72Cl2N6O12P [M+H]+ 826.4769, found: 826.4762.

4.1.4.2. [2-Fluoro-9-(β-D-arabinofuranosyl)adenine]-5'-[(1,2-di-o-palmitoyl-sn-glycerol-3-y]phosphate, sodium salt (30). H NMR (DMSO-d6): δ 8.17 (s, 1H, H-8), 7.78 (br s, 2H, NH2), 6.08 (d, 1H, J1,2 = 5.0, H-1'), 5.87 (d, 1H, J1,2 = 5.0, H-1'), 5.04 (m, 1H, H-2'), 4.27 (d, 1H, J1,1 = 12.0, J1,2 = 3.0, H-1 Gly), 4.16 (m, 1H, H-2', H-3'), 3.82 (m, 1H, H-4'), 3.71 (m, 2H, 2-0H Gly), 2.23 (m, 4H, 2CH2(CH2)3CH3CO), 1.47 (4H, 2CH2(CH2)3CH3CO), 1.28-1.21 (28H, 2CH3(CH2)2CO), 0.85 (t, 6H, 2CH3(CH2)2CO). 13C NMR (Et2N/HCl-salt; DMSO-d6): δ 172.49, 172.25 (2CH3(CH2)2CO), 158.54 (d, JCF3 = 203.0, C(2)), 157.39 (d, JCF3 = 21.5, C(6)), 150.51 (d, JCF3 = 20.5, C(4)), 140.50 (C(5)), 116.46 (d, JCF3 = 4.0, C(5')), 83.67 (C(1')), 82.62 (d, JCF3 = 6.5, C(4')), 75.25, 75.13 (C(2'), C(3')), 70.31 (d, JCF3 = 5.0, C(5)), 64.10 (br s, C(5)), 62.51 (d, JCF3 = 4.0, 62.19 (C-1, C-3 Gly), 54.28 ((CH2)2CH2N), 34.02, 33.83, 31.74, 29.84, 29.54, 29.38, 29.19, 28.97, 24.70, 2.22, 20.83, (CH2)2CH2N), 13.95 (CH3(CH2)2CO), 8.35 ((CH2)2CH2N). 31P NMR (DMSO-d6): δ = -0.51. 31P NMR (DMSO-d6): δ = -52.76. HRMS (ESI): m/z, calcd for C3H6O3N6O12P [M+Na+2H]+ 916.5576, found: 916.5552.

For proteins precipitation, 50% aqueous methanol (50 µL) and acetonitrile (250 µL) were added to each sample (100 µL). The resulting mixture was centrifuged for 10 min at 6500g, and the supernatant was analyzed by HPLC.

The serum samples from the blood of untreated animals were prepared in the same way. The solutions of F-ara-A for calibration curves were prepared by dissolving the nucleoside in the serum of untreated animals. HPLC analysis of the solutions of compound 29 in the serum of untreated mice has demonstrated a stability of the conjugate during 48 h.

The concentration of F-ara-A in serum samples (5 µL) was determined using Waters HPLC system (USA) with UV detector. The separation was performed on Nucleodur EC C18 (250/4.6, 100-5) column at a flow rate of 0.7 mL/min, 40 °C, with isocratic elution (methanol/0.1 M KH2PO4, 20:80).

The pharmacokinetic parameters were calculated using GraphPad Prism 5.0 program (Graph Pad Software Inc., USA). The values of Cmax, Cmin, Tmax, 1/2, and AUC0–24 were expressed as means ± SD (standard deviation).

Experimental results were analyzed with Student's t-test and the obtained values were considered significant versus the F-ara-AMP when P values were lower than 0.05.

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Supplementary data

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References and notes
