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Synthesis and biological evaluation of two glycerolipidic prodrugs of didanosine for direct lymphatic delivery against HIV

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Abstract—Novel glycerolipidic prodrugs of didanosine and didanosine monophosphate designed to by-pass the hepatic first pass metabolism were synthesized and tested for their cytotoxicity and anti-HIV-1 activity. Formulation as liposomes of dipalmitoylphosphatidylcholine was elaborated. A simple quantitative HPLC-UV method was developed and validated, and ESI-MS was used for qualitative purpose. These two prodrugs exhibited promising biological activities against HIV-1 in in vitro infected cell culture.

Didanosine (ddI) (**1**) is a nucleosidic analog for the oral treatment of AIDS, currently marketed as Videx®, and usually used in combination with other antiviral therapy. Didanosine is phosphorylated in vivo to active metabolites that compete for incorporation into viral DNA. These latter inhibit the HIV reverse transcriptase enzyme competitively and act as a chain terminator of DNA synthesis. Didanosine is well tolerated with chronic administration, and its toxicity is uncommon and usually reversible. However, it suffers from a poor bioavailability (20–40%).[1] This drawback is mainly due to acidic degradation in the stomach, and to a mediocre absorption because of its hydrophily and to the hepatic first pass metabolism. Moreover, once in the cell, ddI needs to be sequentially phosphorylated to the 5′-triphosphate by host cell kinases to be active. The first phosphorylation is a limiting step.[2] Glycerolipidic prodrugs, by mimicking long chain triglycerides, are known to enhance the enterocyte absorption and lymphatic transport, therefore they by-pass the hepatic first pass metabolism, hence enhancing bioavailability.[3] Despite the fact that a variety of antiviral or antitumor nucleosides conjugated with lipid moieties have been reported previously,[4] to the best of our knowledge no attempts were made to take advantage of the triglyceride metabolism to improve the bioavailability of ddI. As a matter of fact, triglycerides are hydrolyzed in the small intestine to the corresponding 2-monoglyceride and fatty acids by pancreatic lipase and co-lipase. These metabolites are absorbed by the enterocytes in which they are re-esterified into triglycerides and packaged into intestinal lipoproteins, the chylomicrons. These latter are finally secreted into mesenteric lymph from where they reach the systemic circulation via the thoracic lymph duct, by-passing the liver.[3] Therefore, we hypothesized that ddI covalently bound to the C-2 position of a lipid may have improved bioavailability. As triglycerides with C_{16} acid chains are predominant in nutriments,[5] we chose to conjugate ddI with a 1,3-dipalmitoyl lipid through a succinate linker bound to the 5′-OH group (prodrug **2**). Furthermore, in an attempt to by-pass the first cellular phosphorylation, we speculated that a lipid conjugated to didanosine 5′-monophosphate through an ether linker such as compound **3** may have an improved activity. Comparing the intracellular activities of the non-phosphorylated and the mono-phosphorylated prodrugs (**2** and **3**) on peripheral

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blood mononuclear cells (PBMC) post-infected by HIV, we will establish if it is possible to by-pass the limiting first-phosphorylation step by host cell kinases (Fig. 1).[2]

Herein, we report the syntheses of lipid conjugates of ddI **2** and **3**, the results of their qualitative and quantitative analysis for quality control of the formulation, and their antiviral activity on in vitro HIV-1 infected cells.

The synthesis of prodrug **2** began with the preparation of the symmetrical 1,3-dipalmitoyl glyceride **5** easily available in three steps from 1,3-dihydroxyacetone dimer (**4**) according to the procedure of McCrae.[6]

Thus, condensation of 1,3-diglyceride **5** with glutaric anhydride in the presence of pyridine provided acid **8** (95% yield). The final coupling of **8** with ddI (**1**) was achieved using EDCI to deliver compound **2** in 61% yield.[7] The synthetic methodology, we have developed for the synthesis of **3** involved the application of phosphoramidite chemistry.[8] Thus, 1,3-dipalmitoyl glyceride **5** was condensed with 6-(tetrahydropyranyloxy)hexanoic acid (**6**) using DCC to furnish the triglyceride **7a** in 61% yield. Deprotection of the THP protecting group turned out to be quite troublesome. For example, aqueous acetic acid deprotection of **7a** gave **7b** in low yield along a large amount of diglyceride **5** presumably resulting from acid catalyzed lactonization of **7a** to caprolactone. The use of PPTS[9] in a refluxing mixture of methanol and ethyl acetate was more rewarding, providing the desired alcohol **7b** in 80% yield. The crucial assembly of ddI (**1**) with **7b** was efficiently performed by sequential treatment of **7b** with 2-cyanoethyl *N*,*N*-diisopropylchlorophoramidite (**9**) in the presence of diisopropylethylamine, then 1*H*-tetrazole and finally ddI.[8b] After oxidative work up with aqueous iodine buffered with pyridine, compound **10** was obtained in 93% yield by purification on silica gel column (EtOAc/MeOH/Et_3N, 6:1:0.5%). The final deprotection of **10** was performed via β-elimination using DBU. A considerable amount of cleavage of the hypoxanthine–sugar bond was observed using hydrochloric acid in the work-up. Less acidic conditions were therefore attempted. In the event, brief exposure of the crude product to diluted acetic acid, followed by chromatographic purification on silica gel column (CH_2Cl_2/MeOH, 11:2), furnished phosphodiester **3** in 73% yield.[10] This material slowly decomposed at room temperature through cleavage of the C-1-hypoxhanthine bond, however no substantial decomposition was observed over several month-storage at −30 °C. Compounds **2** and **3** were characterized by ^{1}H NMR, IR, and ESI mass spectrometry. The purity evaluated by HPLC using UV detection and by ESI-MS was estimated to be 98% for compound **2** and 90% for compound **3** (Scheme 1).[11] The quantitative method was validated according to the ICH recommendations in order to undertake quality control of the formulations.[12] The method proved to be accurate (bias <1.4%), with a good repeatability (Relative Standard Deviation (%RSD) <2.9%), and intermediate precision (%RSD <4.2%) for the determination of both compounds. By using HPLC-UV dosage methods, we evidenced the very poor solubility of these prodrugs in water (⩽10.5 μg/mL in water at neutral pH) and their good entrapment efficiency into multilamellar liposomes of dipalmitoylphosphatidylcholine (DPPC)[13] which were prepared according to the Bangham method.[14] These liposomes (prodrugs/DPPC: 1:2 (mol:mol)), whose sizes were evaluated to 1160 ± 129 nm for both compounds, were used in the antiviral assays.

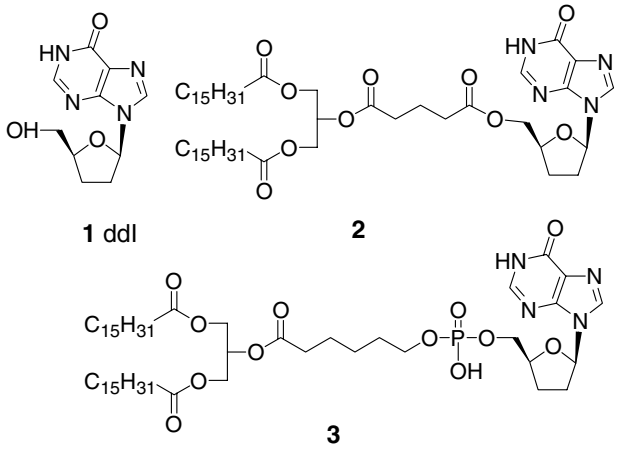
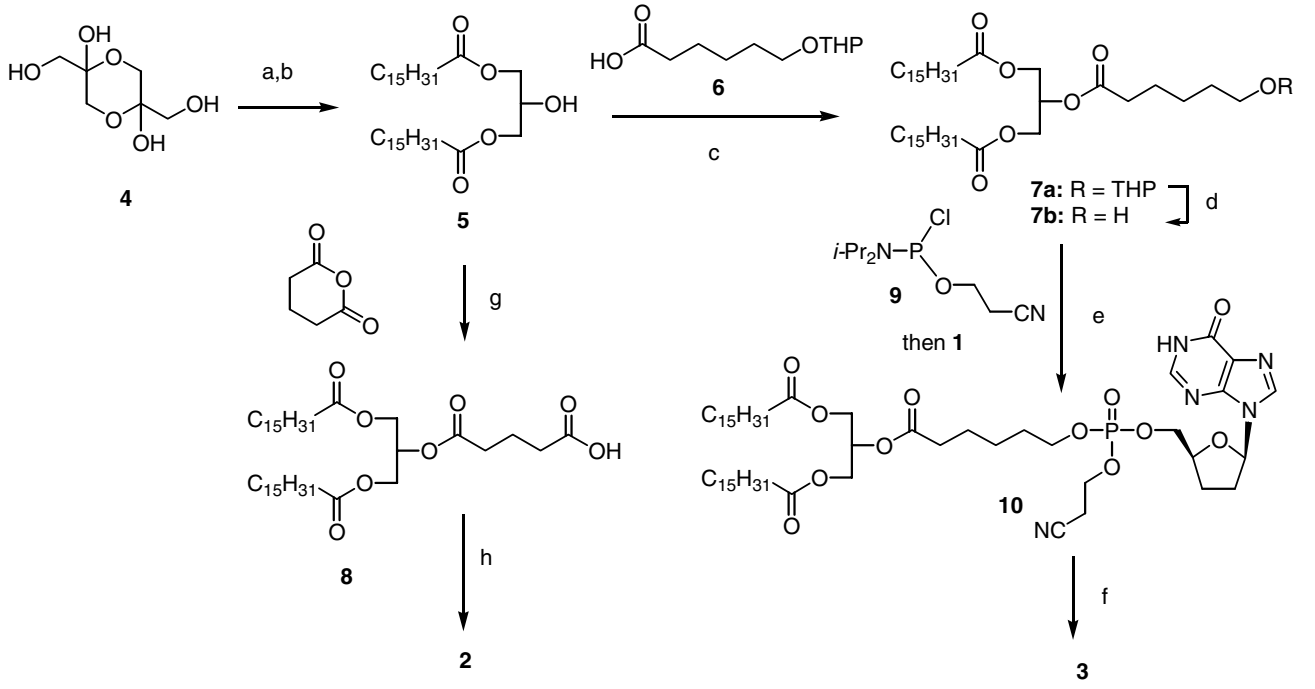


Figure 1. Chemical structures of didanosine (ddI) **1** and lipid conjugates **2** and **3**.

Antiviral assay. In vivo after oral administration, the prodrugs should be modified by digestion, so they will not be in direct contact with infected cells. The antiviral assays were made here to check that the prodrug would be able to deliver ddI in cellular context.

The toxicities assays were performed to ensure that there was no increase in toxicity due to the chemical modification and formulation (previous assays with prodrugs in suspension evidenced cell toxicity by precipitation of prodrugs in culture medium). Phytohemagglutinin-P (PHA-P)-activated peripheral blood mononuclear cells (PBMC) treated by six concentrations (50, 17, 6, 2, 1, and 0.2 μM for compounds **1** and **3**, and 12, 4, 1.3, 0.5, 0.1, and 0.05 μM for compound **2**) of each prodrug as liposomes and, one hour later, were infected with hundred 50% tissue culture infectious doses (TCID50) per 100,000 cells of the HIV-1-LAI strain.[15] This virus was amplified in vitro on PHA-P-activated PBMC.[16] Viral stock was titrated using PHA-P-activated PBMC, and 50% TCID50 were calculated using Kärber's formula.[17] Samples were maintained throughout the culture, and cell supernatants were collected at day 7 post-infection and stored at −20 °C. Viral replication was measured by quantifying reverse transcriptase (RT) activity in cell culture supernatants. In parallel, cytotoxicity of the compounds was evaluated in uninfected PHA-P-activated PBMC by MTT assay on day 7. Experiments were performed in triplicate and repeated with another blood donor. Data analyses were performed using SoftMax®Pro 4.6 microcomputer software: percent of inhibition of RT activity or of cell viability were plotted vs concentration and fitted with quadratic curves; 50% and 90% effective doses (ED_{50} and ED_{90}) and cytotoxic doses (CD_{50} and CD_{90}) were calculated and compared between ddI and ddI prodrugs (Table 1).



Scheme 1. Synthesis of compounds **2** and **3**. Reagents and conditions: (a) $C_{15}H_{31}COCl$, py, $CHCl_3$, cat. DMAP, 3 h, 20 °C, 50%; (b) $NaBH_4$, THF/PhH/H_2O, 5 °C, 30 min, 70%; (c) 1.6 equiv **6**, DCC, DMAP, CH_2Cl_2/THF, 20 °C, 48 h, 61%; (d) 1 equiv PPTS, MeOH/AcOEt, 50 °C, 3 h, 80%; (e) i—1.8 equiv **9**, *i*-Pr_2NEt, CH_3CN, 20 °C, 30 min; ii—5 equiv 1*H*-tetrazole, 1.5 equiv ddI (**1**), THF, 20 °C, 1 h; iii—I_2, THF, Py, H_2O, hexane, 20 °C, 30 min, 93%; (f) i—1.9 equiv DBU, THF, 1 h, 20 °C; ii—AcOH, H_2O, 68%; (g) 2 equiv glutaric anhydride, py, DMAP, THF/CH_2Cl_2, 6 h, 20 °C, 95%; (h) 1.2 equiv EDCI, DMAP, DMF, 50 °C, 3 days, 61%.

Table 1. Anti-HIV-1-LAI effects and cytotoxicity of ddI conjugates **2** and **3** as compared to didanosine (**1**) on activated PBMC in vitro infected by HIV-1-LAI[13]

Compound	ED_{50} (μM)	ED_{90} (μM)	CD_{50} (μM)	CD_{90} (μM)
1 (ddI)	1.2	4.8	40	>50.0
2	2.8	>12.0	>12.0	>12.0
3	28.5	>50.0	>50.0	>50.0

Results are expressed as ED_{50} and ED_{90}, concentration of drugs that decreases the HIV replication of 50% and 90%, respectively (means of ED from two blood donors). CD_{50} and CD_{90}, concentration of drugs to reduce the viable cell number by 50% and 90%, respectively (means of ED from two blood donors).

The data indicated that **2** was roughly equi-active with ddI against HIV-1. **3** was a little less active, probably due to the ionized phosphate which could slow down cellular absorption and might hide the by-pass of the first phosphorylation. Another alternative hypothesis is that the prodrug would not release the monophosphate of ddI because aliphatic phosphate esters are known to be difficult to be cleaved enzymatically. Further experiments would be performed in order to study these hypotheses. However, these two new compounds showed no sign of increased cytotoxicity at tested doses (12 and 50 μM for **2** and **3**, respectively). Both compounds appear promising ddI prodrugs because they keep an anti-HIV activity and would exhibit a better bioavailability than ddI after oral administration.

In conclusion, we have synthesized with good purity two novel didanosine derivatives as prodrugs compounds active against HIV-1 in infected cell culture with no sign of increased toxicity. Since these molecules were poorly water soluble, adapted liposomal formulation was developed. A simple dosage method with good accuracy, repeatability, and intermediate precision was elaborated in order to evaluate these formulations. Further experiments including absorption studies in in vitro CaCo-2 cells culture of these two new molecules in liposomes are currently performed in comparison with ddI to validate the here presented biomimetic strategy.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.01.062.

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7. Characterization data for compounds **2**: colorless oil, $[\alpha]_D^{20} = -7$ (*c* 0.7, $CHCl_3$); IR (neat, cm^{-1}) *v* 3600–2500, 1617, 1588, 1544; ^1H NMR ($CDCl_3$, 1% D_2O, 300 MHz) δ 13.0 (br s, 1H), 8.15 (s, 1H), 8.00 (s, 1H), 6.21 (t, $J = 4.6$ Hz, 1H), 5.17 (quint, $J = 5.0$ Hz, 1H), 4.40–4.15 (m, 5H), 4.07 (dd, $J = 12.0$, 5.8 Hz, 2H), 2.55–2.45 (m, 2H), 2.36 (t, $J = 7.3$ Hz, 2H), 2.33 (t, $J = 7.4$ Hz, 2H), 2.23 (t, $J = 7.6$ Hz, 4H), 2.18–1.95 (m, 2H), 1.86 (quint, $J = 7.4$ Hz, 2H), 1.60–1.48 (m, 4H), 1.30–1.10 (m, 48H), 0.80 (t, $J = 6.9$ Hz, 6H).
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10. Characterization data for compounds **3**: colorless oil, $[\alpha]_D^{20} = -13$ (*c* 1.1, $CHCl_3$); IR (neat, cm^{-1}) *v* 3600–2500, 2955, 2916, 2850, 1736, 1693, 1588, 1468, 1219, 1095, 1063; ^1H NMR ($CDCl_3$, 1% D_2O, 300 MHz) δ 8.50 (s, 1H), 8.07 (s, 1H), 6.26 (m, 1H), 5.20 (q, $J = 5.5$ Hz, 1H), 4.50–4.20 (m, 5H), 4.12 (dd, $J = 11.9$, 5.4 Hz, 2H), 4.02–3.80 (m, 2H), 2.60–200 (m, 4H), 2.31 (t, $J = 7.5$ Hz, 6H), 1.60–1.48 (m, 6H), 1.30–1.10 (m, 52H), 0.84 (t, $J = 6.5$ Hz, 6H); ^{31}P NMR ($CDCl_3$, 81 MHz) δ 1.8 (broad s).
11. HPLC-UV characteristics: Nucleosil® C18 column, 10 μm, 250×4 mm. Elution with CH_3OH/CH_3CN/0.05% aqueous ammonium acetate (v/v/v) pH 8 (97.5/1.5/1.5; v/v/v) for **2** and (87.5/5/7.5; v/v/v) for **3** at 1 mL/min. Absorption maxima = 280 nm for **2** in methanol and 254 nm for **3**. Compound **2**: peak at 13.6 min corresponding to Mw = 900.6 (*m/z* 901.6, $[M+H]^+$ and *m/z* 923.6, $[M+Na]^+$, positive ESI mass spectrum). Compound **3**: main peak at 9.8 min, corresponding to Mw = 980.6 (*m/z* 979.6, $[M-H]^-$, negative ESI mass spectrum) with a minor impurity at 9.1 min (underprotected phosphotriester **10** (*m/z* 1032.6, $[M-H]^-$)).
12. ICH (Q2A) and (Q2B): (CPMP/ICH/381/95), (CPMP/ICH/281/95), 1994. Calibration curves = linear response (10–100 μg/mL) ($r^2 \geqslant 0.995$). Limits of detection/quantification = 2.6/7.8 μg/mL for **2** and 1.2/3.7 μg/mL for **3.** For each sample, $n = 6$.
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