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Metabolism evaluation of biomimetic prodrugs by *in vitro* models and mass spectrometry

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ABSTRACT

Glycerolipidic prodrug is an interesting concept to enhance lymphatic absorption of polar drugs intended to oral delivery such as didanosine (ddI). In order to improve ddI bioavailability, two didanosine glycerolipidic prodrugs, the phosphorylated (ProddIP) and the non-phosphorylated derivatives (ProddINP) were synthesized to follow triglyceride metabolism. The biomimetism approach of these prodrugs has been studied *in vitro* at two steps. First, liposomal formulation of each prodrug was incubated with a lipolysis model based on pancreatin and analysed using liquid chromatography combined with tandem mass spectrometry (LC–MS/MS). These experiments evidenced that both didanosine prodrugs were recognized by the lipases; as expected, they were cleaved at both positions *sn*-1 and *sn*-3 of glycerol. ProddIP was metabolised twice more rapidly than ProddINP suggesting an implication of some phospholipases in ProddIP degradation. Secondly, the detection of dideoxyadenosine triphosphate (ddA-TP) into HIV-1 infected cells after their incubation with ProddINP loaded liposomes evidenced their ability to release ddI that could penetrate into the cells and be metabolised by intracellular kinases. These results confirmed that the synthesized glycerolipidic prodrugs of didanosine could be investigated for a biomimetic approach with final aiming of increasing the drug oral bioavailability by enhancing intestinal absorption.

1. Introduction

Didanosine (ddI) is a nucleoside reverse transcriptase inhibitor (NRTI), actually commercialised as Videx®. It is usually used in combination with other antiviral agents in the HAART (highly active antiretroviral therapy) against HIV-1 infection in adults (Yatvin et al., 1999). ddI requires a metabolisation step and three phosphorylation steps, catalysed by cell kinases like all NRTIs, in order to be converted into its active triphosphate metabolite, dideoxyadenosine triphosphate (ddA-TP) (Tan et al., 1999) (Fig. 1). This latter then acts as competitive inhibitor or alternative substrates with respect to the normal substrates and leads to the termination of chain elongation of viral DNA (De Clercq, 2007). However, oral bioavailability of didanosine could be reduced by degradation in the acidic medium of the stomach, first-pass hepatic metabolism and poor intestinal membrane permeation (Aungst, 1999; Yatvin et al., 1999).

The synthesis of lipid based prodrugs is an approach to improve drug bioavailability via oral route (Charman and Porter, 1996; Porter and Charman, 1997; Delie et al., 1994). Glyceridic prodrugs like triglyceride analogues can be used for such purposes. By substituting one or more fatty acids of a triglyceride by a drug molecule, this glycerolipidic prodrug would follow the metabolic pathways of these natural lipids (Garzon-Aburbeh et al., 1986; Porter and Charman, 1997). Physiologically, triglycerides with long chain fatty acids are hydrolyzed in the small intestine to the corresponding 2-monoglyceride and fatty acids by pancreatic lipase and co-lipase (Fig. 2a). These metabolites are absorbed by the enterocytes in which they are reesterified into triglycerides and packaged into the chylomicrons. These intestinal lipoproteins are finally secreted into

Abbreviations: CID, collision-induced dissociation; ddI, didanosine or 2′,3′ dideoxyinosine; ddA-TP, dideoxyadenosine triphosphate; Diglyceryl- and 2-monoglyceryl-ProddINP, diglyceryl- and 2-monoglyceryl-metabolites of ProddINP; Diglyceryl- and 2-monoglyceryl-ProddIP, diglyceryl- and 2-monoglyceryl-metabolites of ProddIP; DPPC, dipalmitoylphosphatidylcholine; LC–MS/MS, liquid chromatography tandem mass spectrometry; MRM, multiple reaction monitoring; ProddINP, non-phosphorylated prodrug of didanosine; NRTI, nucleoside reverse transcriptase inhibitor; ProddIP, phosphorylated prodrug of didanosine; QELS, quasielastic laser light scattering.

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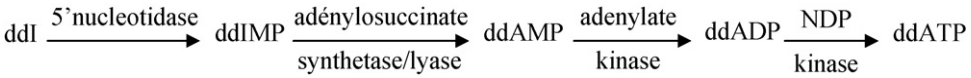

Fig. 1. Intracellular metabolism of didanosine: didanosine monophosphate (ddIMP), dideoxyadenosine monophosphate (ddAMP), dideoxyadenosine diphosphate (ddADP), and dideoxyadenosine triphosphate (ddA-TP).

mesenteric lymph from where they can reach the systemic circulation via the thoracic lymph duct, thus, by-passing the liver (Drusano et al., 1992; Porter and Charman, 1997). By following the similar triglyceride metabolism pathway thanks to glycerolipidic prodrug, the drug bioavailability is improved by enhancing the enterocyte absorption and lymphatic transport, therefore by-passing the hepatic first pass metabolism (Porter and Charman, 1997; Lambert, 2000).

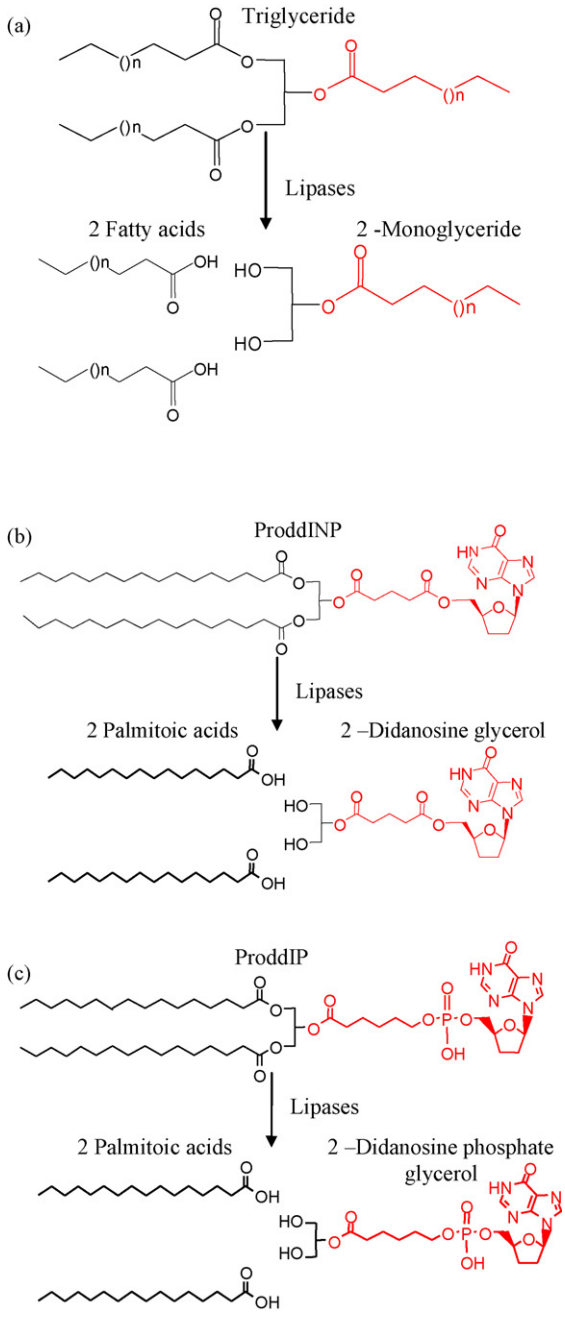

Fig. 2. Metabolism of (a) long chain fatty acid triglyceride and glycerolipidic prodrugs, (b) ProddINP and (c) ProddIP by lipases (pancreatin lipase and co-lipase).

The use of lipidic formulations is a well known strategy to enhance hydrophobic drug solubility in the goal of oral administration (Pouton, 2000; Christensen et al., 2004). These formulations consist of a mixture of the drug and different types of lipids, such as triglycerides and phospholipids. Herein, the intestinal absorption of the drug is enhanced through the formation of mixed micelles composed of the lipolysis products and the drug (Pouton, 2000; Porter et al., 2007). Moreover because of their rich lipid composition, these formulations generally increase chylomicron synthesis by the enterocytes and enhance lymphatic absorption (Porter et al., 2007).

To address the problem of ddI bioavailability, two glycerolipidic prodrugs, a phosphorylated one (ProddIP) and a non-phosphorylated one (ProddINP) were synthesized (Lalanne et al., 2007a). The mono-phosphorylated prodrug (ProddIP) is designed in order to by-pass the first cellular phosphorylation step. We chose to conjugate ddI or ddI 5′ monophosphate with a 1,3-dipalmitoyl diglyceride through a linker bound to the 5′-OH group. It was to note the *sn*-2 position of ddI or ddI monophosphate in these prodrugs. Both prodrugs were prepared in a lipid-based formulation composed of dipalmitoylphosphatidylcholine (DPPC) which has a relative structural analogy with prodrugs as demonstrated by previous studies (Lalanne et al., 2007b; Bourgeois et al., 2009).

This biomimetic approach would increase enterocyte absorption and lymphatic transport and avoid the hepatic first pass metabolism, hence leading to increase the bioavailability and the efficacy of didanosine. The first step in the metabolism of these prodrugs by oral route could be the degradation by pancreatic lipase and co-lipase according to Fig. 2. This enzymatic reaction has been demonstrated to be kinetically controlled by the spacer used in the lipid prodrugs (Scriba, 1993; Lambert, 2000).

The purpose of the present study was to validate the metabolic pathway of the biomimetic strategy based on glycerolipidic prodrug. The biomimetism approach of these glycerolipidic prodrugs has been studied *in vitro* at two levels: the prodrug metabolism by enzymes of gastrointestinal (GI) tract and the final metabolism step by intracellular kinases into HIV-1 infected cells. First, to study the biotransformation kinetic of glycerolipidic prodrugs by gastrointestinal enzymes, a lipolysis model based on pancreatin and an extraction method of prodrugs and metabolites were developed and evaluated. A high performance liquid chromatography combined with tandem mass spectrometry (LC–MS/MS) method was investigated in order to identify the metabolites, to separate each prodrug and its corresponding metabolites and to follow their concentration evolution as a function of incubation time. An additional experiment was performed to study the ability of didanosine prodrugs mixed with lipids to release ddI that could be metabolised by intracellular kinases. The presence of the active metabolite of didanosine, ddA-TP, into HIV infected cells was evaluated after incubation of prodrugs liposomal formulations and quantified by LC–MS/MS.

2. Materials and methods

2.1. Chemicals

Didanosine was purchased from Bristol Myers Squibb (NY, USA). ProddINP and ProddIP prodrugs were synthesized by Artmolecules (Poitiers, France) as described by Lalanne et al.

(2007a). 2-Chloroadenosine 5′-triphosphate (ClATP) and N′,N′-dimethylhexylamine (DMH) were provided by Sigma–Aldrich (St Louis, MO, USA). Dideoxyadenosine triphosphate (ddA-TP) was synthesized by C. Guerreiro and R. Sarfati of the Unité de Chimie Organique, Institut Pasteur, Paris, France.

Dipalmitoylphosphatidylcholine (DPPC), L-α-lecithin (L-α-phosphatidylcholine, EPC) type X-E from dried egg yolk, sodium taurodeoxycholate (NaTDC), tributyrin, calcium chloride (USP specification), trizma® maleate and pancreatin from porcine pancreas (activity equivalent to 8× USP specifications) were purchased from Sigma (St Louis, MO, USA). Sodium hydrogenophosphate (Na_2HPO_4) and sodium dihydrogen phosphate (NaH_2PO_4) were obtained from VWR Prolabo (Fontenay sous Bois, France). HPLC grade methanol and acetonitrile were provided by Carlo Erba (Rodano, Italy), formic acid by Merck (Darmstadt, Germany). Ultrapure water was prepared using a Milli-Q™ system (Millipore, St Quentin-en-Yvelines, France).

2.2. Characterization of didanosine prodrugs by mass spectrometry

Both prodrugs were characterized by their molecular mass and by their product ion mass spectra. Mass spectrometry (MS) scan and daughter scan (MS/MS) analyses, in the positive ionisation mode (optimised for both prodrugs), were achieved by direct infusion of 20 μM of ProddINP or ProddIP diluted in methanol/water pH 7.5 (95/5, v/v) or methanol/water set at pH 7.5 (85/15, v/v), respectively. Collision-induced dissociation (CID) was optimised to 25 eV by increasing collision energy between 5 and 30 eV, in order to produce intense product ions while detecting the parent ions at *m*/*z* 901.4 (protonated ProddINP) and *m*/*z* 981.6 (protonated ProddIP) with 5% relative abundance.

2.3. Formulation of didanosine prodrugs

2.3.1. Formulation of ProddINP in liposomes

The liposomes were prepared by a solvent emulsion–evaporation technique avoiding filtration step and loss of ProddINP on filters. Briefly, DPPC (3.25 mg) was dissolved with ProddINP (0.4 mg) in chloroform (0.4 mL). Then 4 mL of Milli-Q water were added. After two cycles of ultrasonication at 450 W, 1 min each, using a Vibra cell sonicator (Bioblock Scientific, France) on ice, the chloroform was completely evaporated using a Rotavapor® to obtain a lipid concentration of 2 mg/mL (Prodrug + DPPC) for a ProddINP:DPPC ratio of 1:10 (mol:mol).

The mean diameter of the prepared liposomes was 187 ± 75 nm as determined by quasielastic laser light scattering (QELS) with a Nanosizer Coulter model N4 MD® (Coulter Electronic, Margency, France).

2.3.2. Formulation of ProddIP in liposomes

Liposomes of ProddIP:DPPC (mol:mol) 1:5 were prepared as described before (Lalanne et al., 2007b). Briefly, the multilamellar vesicles (MLV) of DPPC/ProddIP were prepared according to Bangham's method (Bangham et al., 1974). ProddIP (0.4 mg) and DPPC (1.5 mg) were dissolved in methanol (0.2 mL) and chloroform (0.1 mL), respectively. After being mixed, the solvents were evaporated under vacuum using a Rotavapor®. The residual traces of organic solvents were evaporated under low vacuum, overnight in a lyophilizer. The lipid film was then rehydrated with heated phosphate buffer at 60 °C ($[HPO_4^{2-}]$ = 48 mM; $[H_2PO_4^-]$ = 28 mM; $[Na^+]$ = 144 mM; $[Cl^-]$ = 20 mM; pH 7.4) to obtain a lipidic concentration of 2 mg/mL (Prodrug + DPPC) for a ProddIP:DPPC ratio of 1:5 (mol:mol). The liposomal suspension was then filtered three times with 0.8 μm filters (Sartorius®, Palaiseau, France). The mean diameter of the prepared liposomes was 777 ± 90 nm as determined by QELS.

2.4. Metabolism of didanosine prodrugs by pancreatin

2.4.1. Incubation of didanosine prodrugs with pancreatin

The enzymatic activity of the *in vitro* model of lipolysis was assessed using similar conditions as previously described (Alvarez and Stella, 1989; MacGregor et al., 1997).

The lipolysis experiments were carried out in the presence of 1 g pancreatin in 9 mL of the digestion buffer (pH 7.5) containing 50 mM of tris maleate, 150 mM NaCl, 5 mM $CaCl_2 \cdot 2H_2O$. The overall mixture was kept 15 min under magnetic stirring, and then centrifuged at 16,000 × *g* at 5 °C. The supernatant was collected and kept on ice until lipolysis assays were started. Fresh pancreatin extracts were prepared each day. Then, sodium taurocholate (20 mM) and egg phosphatidylcholine (5 mM) were added to the supernatant.

Lipase activity was expressed in terms of tributyrin units (TBU), where 1 TBU is the amount of enzyme that can liberate 1 μmol of fatty acid from tributyrin per min. NaOH (1 M) was used as the titration solution for enzyme activity determination. Therefore, *in vitro* lipolysis assays were performed at 37 °C under magnetic stirring, and were initiated by the addition of the lipid-based formulation of the prodrugs to the reaction mixture (1/1, v/v). The onset of lipid digestion results in the release of fatty acids, which causes a transient drop in pH. This pH is maintained between 6.5 and 8.5 during the experiment by adding ammonia (2.5%), thereby allowing the pH-sensitive process of digestion to continue with an optimum lipase activity as reported by Gargouri et al. (1989).

Throughout the lipolysis process, aliquots were taken at *t* = 0.5, 5, 10, 15, 30 and 60 min. The lipolysis reaction was stopped by enzyme inactivation with the extraction solvent. The prodrugs and their metabolites were then extracted from the lipolysis medium.

2.4.2. Extraction of the didanosine prodrugs and their corresponding metabolites

Prodrugs and their corresponding metabolites were extracted from the enzymatic medium using Folch extraction method described for the extraction of total lipids from animal tissues (Folch et al., 1957). Briefly, 50 μL of the lipolysis mixture were added to 950 μL of chloroform/methanol (2/1, v/v) mixture. The whole mixture was kept for 10 min in an ultrasonic bath at room temperature. Then the mixture was centrifuged 15 min at 10,000 × *g* at 4 °C (Eppendorf centrifuge 5415R). The supernatant was collected and evaporated under nitrogen flow. The residues were kept at −20 °C until analysis. Prior to the analysis, the residues were dissolved with 500 μL of the appropriate mobile phase and submitted for 10 min in an ultrasonic bath at room temperature. 20 μL were analysed by HPLC/UV. The efficacy of extraction method was carried out on inactivated pancreatin in the presence of all the components used in lipolysis medium, spiked with the formulated prodrug. Recovery values for both compounds were measured by the HPLC-UV method described by Lalanne et al. (2007a). Recoveries were calculated by comparing the recovered concentrations of each prodrug to those of untreated liposomes (three replicates).

2.4.3. LC–MS/MS of didanosine prodrugs and their metabolites

The chromatographic conditions were adapted from previously analytical HPLC-UV methods of ddI prodrugs (Lalanne et al., 2007a). Glycerolipidic prodrugs and their enzymatic metabolites were analysed by liquid chromatography and tandem mass spectrometry (LC–MS/MS) using a 1100 series HPLC system (Agilent Technologies, Massy, France) including an autosampler, a binary pump and an Uptisphere® C18 column 3 μm, 2 mm i.d. × 50 mm length (Interchim, Montluçon, France). For ProddINP, isocratic elution was achieved with methanol/5 mM ammonium formate pH 7 (98/2,

v/v) at a flow rate of 0.25 mL/min. The total analysis time was 15 min per sample. For ProddIP, isocratic elution was achieved with methanol/acetonitrile/0.05% ammonium acetate; pH 8 (87.5/5/7.5, v/v/v) at a flow rate of 0.2 mL/min. The total analysis time was 10 min per sample. Prior to each sample injection, the autosampler needle was washed twice with 30 μL of the mobile phase.

Detection was performed with a Quattro®-LCZ triple quadrupole mass spectrometer equipped with the orthogonal electrospray source (Waters Micromass, Manchester, UK). Analytes were detected in the positive ion mode using tandem mass spectrometry with multiple reaction monitoring mode (MRM) using a dwell time of 0.5 s. The capillary voltage was set at 3500 V. The source temperature and the nebulization gas temperature were set at 90 and 200 °C, respectively. Nitrogen gas flow was set at 450 l/h. Collision gas (argon) pressure was set at 1.3 mbar. Cone voltages were set at 25 V for both ProddINP and ProddIP analyses.

Collision energies and transitions ion pairs were optimised for each analyte by infusion at 10 μl/min with a Harvard syringe pump. MRM transitions and daughter scans for the detection of ProddINP and ProddIP were performed with collision energy of 25 eV. Data were processed using MassLynx™ software (Waters Micromass, Manchester, UK).

The specificity of the analytical method was studied by analyzing a blank sample containing pork pancreatin extracts by LC–MS/MS conditions used for the detection of each prodrug and its corresponding metabolites. The detection method was selective using the combination of specific MRM transitions and retention times of each analyte. No significant interference in the different blank pancreatin samples was found at the retention times of ProddINP and ProddIP and of their corresponding metabolites for each MRM transition, respectively. Noteworthy, metabolites were characterized with m/z $[M+H]^+$ or $[M+Na]^+$ ions.

Concentration versus incubation time curve was established for each prodrug. The relative transformation rate of the prodrug was defined as the quantity of consumed prodrug per minute as calculated from the concentration versus time curve. For metabolites, peak areas (arbitrary unit) were plotted as a function of time. It was to note that during this experiment, the error on peak area was around 2000 AU.

2.5. Evaluation of didanosine prodrugs intracellular metabolism into HIV-1 infected cells

2.5.1. Incubation of didanosine prodrugs with HIV-1 infected cells

Phytohemaglutinin-P (PHA-P)-activated peripheral blood mononuclear cells (PBMCs) were treated by three concentrations of didanosine and prodrugs loaded liposomes: 20, 10, 1 μM. One hour later, cells were infected with hundred 50% tissue culture infectious doses (TCID50) per 100,000 cells of the HIV-1-LAI strain (Barre-Sinoussi et al., 1983). This virus was amplified *in vitro* on PHA-P-activated PBMC (Roisin et al., 2004). Viral stock was titrated using PHA-P-activated PBMC, and 50% TCID50 were calculated using Kärber's formula (Kärber, 1931). 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. Experiments were performed in triplicate.

2.5.2. Quantification of ddA-TP in HIV-1 infected cells by LC–MS/MS

After 24 h incubation of prodrugs, PBMCs were spiked with 20 μL of the internal standard ClATP (c = 160 ng/ml) according the previously described procedure (Becher et al., 2002). Then, ddA-TP was extracted using 500 μL cold Tris–HCl (0.05 M: pH 7.4) and methanol (70:30, v/v) and quantified using the improved validated LC–MS/MS assay (Pruvost et al., 2005). The limit of quantification (LOQ) of ddA-TP is 25 fmol per sample, using the following conditions.

Briefly, the chromatographic conditions were used from a previously developed LC–MS/MS assay of intracellular NRTI-TPs (Henneré et al., 2003). ddA-TP and the internal standard (ClATP) were analysed by LC–MS/MS using a 1100 series HPLC system (Agilent Technologies, Massy, France) including an autosampler, a binary pump and a Supelcogel® ODP-50, 150 mm × 2.1 mm, 5 μm particle size column (Supelco, St. Quentin-Fallavier, France). A gradient elution was carried out with eluent A (water/6 mM DMH, 20 mM ammonium formate pH 5 (50:50, v/v)) and with eluent B (acetonitrile/eluent A (50:50, v/v)). Gradient elution was as follows: after initial conditions with A and B (70:30) for 2 min, a linear gradient was performed from 70:30 to 35:65 between 2 and 12 min and then up to 10% B from 12 to 13 min over 3 min. An equilibration time of 10 min was set before next analysis. The flow rate was set at 0.3 mL/min at a temperature of 30 °C. The total run time was 25 min per sample.

Detection was achieved using a Quantum Discovery® triple quadrupole tandem mass spectrometer (Thermo-Electron, Les Ulis, France) operating with an electrospray source in the negative ion mode using multiple reaction monitoring mode (MRM). The ionspray voltage was set at −3300 V and the capillary temperature at 330 °C. Nitrogen was used as a nebulising gas set at 44 l/h and argon as collision gas. The MRM transitions monitored at a dwell time of 0.5 s were 474 → 159 and 540 → 159 for ddA-TP and ClATP, respectively. Cone voltages were set at −103 V and −133 V, with collision energies of −28 and −36 V, respectively. Data were processed using Excalibur™ software (Thermo-Electron, Les Ulis, France).

3. Results

Two metabolism experiments have been performed on didanosine glycerolipidic prodrugs.

First, the biotransformation kinetic of glycerolipidic prodrugs by pancreatin was studied by an optimised LC–MS/MS method. Secondly, the presence of the active metabolite of didanosine, ddA-TP, into HIV infected cells was evaluated after incubation of prodrugs liposomal formulations by a previously validated LC–MS/MS assay (Becher et al., 2002).

3.1. Metabolism of didanosine prodrugs by pancreatin

3.1.1. Validation of lipolysis experiments

First the lipase activity of pancreatin extract used in these experiments has been measured. The pancreatic lipase activity in the presence of the prodrugs was 48 TBU/ml, in agreement with the reported human gastrointestinal lipase activities (8–50 TBU) (Embleton and Pouton, 1997). Then, in order to validate the biomimetic strategy, it was important to extract all the analytes from the enzyme rich medium. For this purpose, the extraction yields of ProddINP and ProddIP were evaluated from the lipolysis medium with inactivated pancreatin lipases using a method adapted from Folch et al. (1957) considering the amphiphilic properties and the high resemblance of these prodrugs with glycerolipids. Different parameters have been studied to optimise the method. The variation of the chloroform/methanol ratio did not significantly increase the recovery yields of each prodrug. On the other hand, the sonication step impressively improved the recoveries of both prodrugs, since it enhanced their dissolution in the chloroform/methanol (2/1) mixture. Using this liquid–liquid extraction, the mean extraction recovery yields of ProddIP and ProddINP from enzymatic rich medium were 96 ± 4% and 100 ± 3%, respectively (n = 3). Since it provided good extraction of prodrugs, this method was used during the lipolysis experiment.

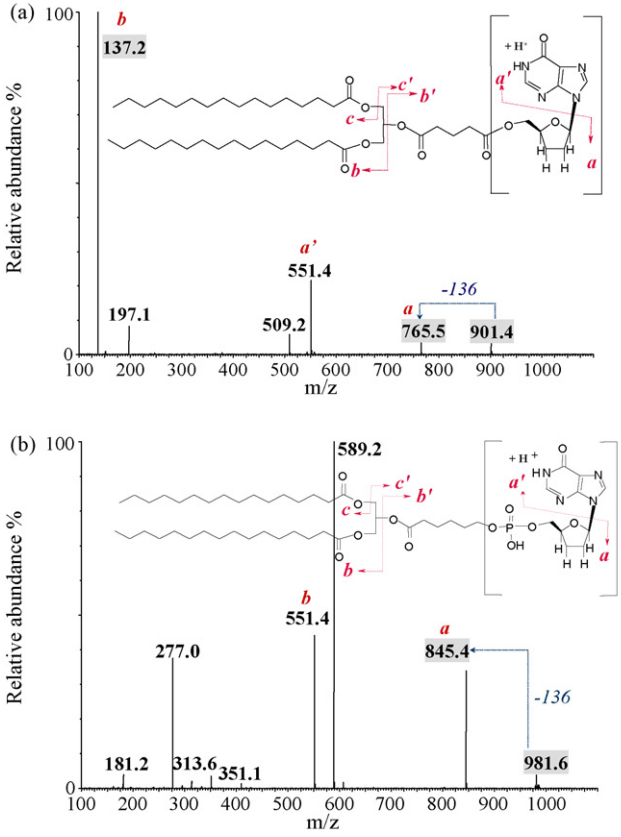

Fig. 3. Product ion mass spectra of (a) ProddINP in MeOH/H_2O pH 7.5 (95/5, v/v) and of (b) ProddIP in MeOH/H_2O pH 7.5 (85/15, v/v).

3.1.2. *Detection and study of prodrugs and metabolites by mass spectrometry*

3.1.2.1. Mass spectrometry study of prodrugs. The product ion mass spectrum in the positive ionisation mode of ProddINP at 25 eV showed the parent ion detected at *m/z* 901.4 and five daughter ions detected at *m/z* 765.5, 551.4, 509.2, 197.1 and 137.2 (Fig. 3a). The product ion mass spectrum of ProddIP in the same conditions showed the parent ion at *m/z* 981.6 and four major daughter ions detected at *m/z* 845.4, 589.2, 551.4 and 277.0 (Fig. 3b). Moreover, the phosphate group present in ProddIP was confirmed by the detection of ProddIP in the negative ionisation mode with $[M-H]^-$ at *m/z* 979.6 (data not shown). The structure and the proposed fragmentation pathways observed for each prodrug are annotated in Fig. 3a and b, respectively. These spectra showed the transitions *m/z* 901.4 → 765.5 and *m/z* 981.6 → 845.5 for ProddINP and ProddIP, respectively which corresponded to the loss of (*m/z* 136) which was specific to the fragmentation of the prodrug didanosine part. These specific MRM transitions were chosen to detect prodrugs in lipolysis experiments.

3.1.2.2. Mass spectrometry study of metabolites. The identification of the metabolites formed after 60 min incubation of each prodrug with lipolysis medium was performed by their product ion mass spectra (Fig. 4).

3.1.2.2.1. ProddINP. According to the expected metabolism pathway (Fig. 2b), we first confirmed the formation of diglyceryl- and 2-monoglyceryl-metabolites of ProddINP by their respective molecular mass (*M* = 664 and 425), as shown in Fig. 4a and b. Then we will keep the respective names: diglyceryl- and 2-monoglyceryl-ProddINP. Daughter scans in the positive ionisation mode of these metabolites produced the formation of the ion *m/z* 137.0. This fragment ion could correspond to a protonated ion of hypoxanthine

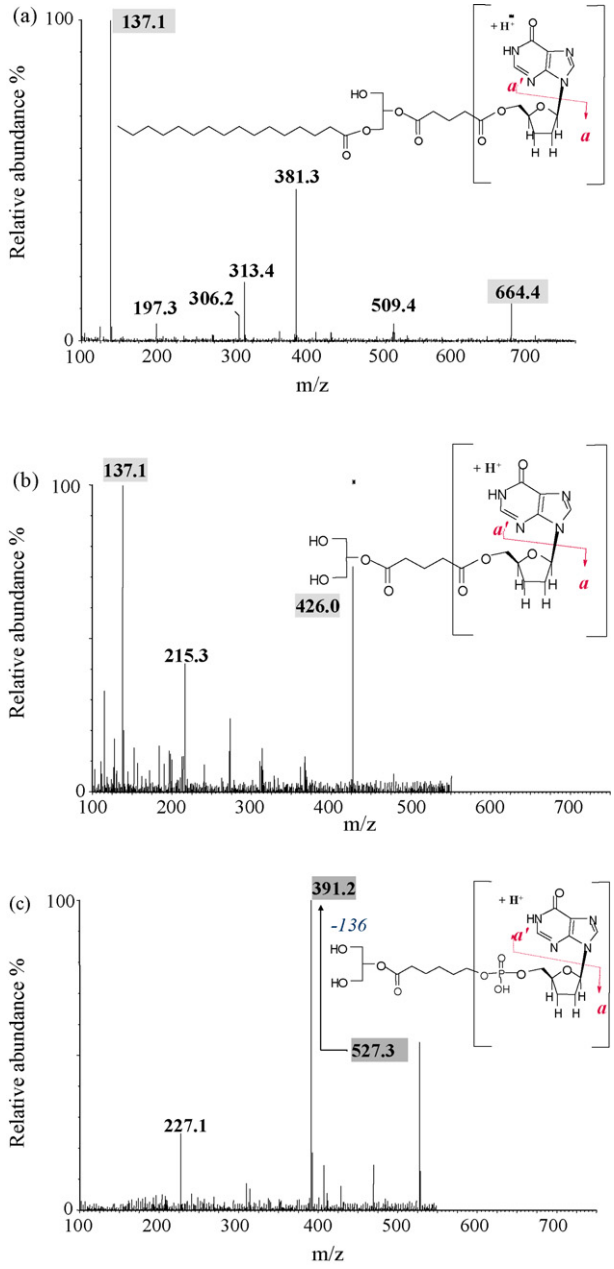

Fig. 4. Product ion mass spectra and proposed structure of the identified metabolites: (a) 2-monoglyceryl-ProddINP, (b) diglyceryl-ProddINP and (c) 2-monoglyceryl-ProddIP extracted from the lipolysis medium after 60 min incubation.

as observed on the product mass spectrum of ProddINP (Fig. 3a). Thus, the mass spectra of the two metabolites showed the same loss of 136 mass and the formation of the product ion *m/z* 137.0 (Fig. 4a and b). Other possible metabolites were not found using LC–MS/MS analyses with MS/MS scans in the positive mode or with MRM transitions between the parent ion and the daughter ion *m/z* 137. Finally, in order to detect 2-monoglyceryl-ProddINP and the diglyceryl-ProddINP during the lipolysis experiment, the following MRM transitions were selected: *m/z* 664.8 ($[M+H]^+$) → 137.0 and *m/z* 425.8 ($[M+H]^+$) → 137.0, respectively.

3.1.2.2.2. ProddIP. Similarly, we investigated the formation of diglyceryl- and 2-monoglyceryl-metabolites of ProddIP called diglyceryl- and 2-monoglyceryl-ProddIP (Fig. 2c). Their respective molecular mass was found (*M* = 742 and 504). Only the 2-monoglyceryl-ProddIP was significantly observed under our

experimental conditions. Daughter scan of m/z 527.3 ($[M+Na]^+$) evidenced the specific loss of 136 mass and thus the formation of the product ion m/z 391.2 (Fig. 4c). This implies that m/z 527.3 corresponds to the 2-monoglyceryl-ProddIP. Therefore, as no other possible metabolite was detected, MRM analyses were based on the following transitions m/z 765.5 → 629.5 and m/z 527.3 → 391.0, to detect digylceryl- and monoglyceryl-metabolites of ProddIP, respectively.

3.1.3. Enzymatic digestion of the prodrugs

3.1.3.1. LC–MS/MS analysis of in vitro lipolysis products. A LC–MS/MS method was developed using MRM for selective detection of ProddIP and ProddINP and their metabolites based on their specific transitions determined by MS/MS analyses. The LC–MS/MS isocratic run allowed the separation of the prodrugs and the metabolites. ProddINP was eluted at 9.7 min (Fig. 5a), the diglyceryl-ProddINP at 1.5 min (Fig. 5b) and the 2-monoglyceryl-ProddINP at 0.7 min (Fig. 5c). ProddIP was eluted at 6.7 min, the diglyceryl-ProddIP at 1.1 min and the 2-monoglyceryl-ProddIP at 0.7 min (data not shown).

3.1.3.2. Lipolysis kinetic of the prodrugs by pork pancreatin.

3.1.3.2.1. ProddINP. Liposomal suspensions of 35 μM ProddINP were incubated with pancreatin and analysed by the LC–MS/MS method. As time of incubation increased, the concentrations of ProddINP in the lipolysis medium decreased (Fig. 5a). In fact, after 10 min of enzymatic incubation, 23% of the initial ProddINP were detected in the lipolysis medium (Fig. 5a; A_4). After 15, 30 and 60 min of enzymatic incubation time, ProddINP initial concentration were detected at 6%, 3% and 1%, respectively (Fig. 5a; A_5–A_7). The concentrations of ProddINP were estimated at each time point and reported as a function of incubation time (Fig. 6a). The relative transformation rate of ProddINP was then estimated around 5 μM/min.

As a function of incubation time of ProddINP with pancreatin, diglyceryl- and 2-monoglyceryl-ProddINP appeared (Fig. 5b and c, respectively). The diglyceryl metabolite was only observed at the beginning of incubation (before 5 min) (Fig. 5b). The amount of monoglyceryl metabolite in the medium increased during incubation between 5 and 30 min (Fig. 5c). The decrease of metabolite amount at 60 min (Fig. 5c; C_7) cannot be explained by another step of metabolism because no more metabolite was detected in our experiments. It was not significant considering the error on peak area observed during these experiments. The evolution of peak areas corresponding to both metabolites as a function of incubation time was shown in Fig. 6b and c.

3.1.3.2.2. ProddIP. Liposomal suspensions of 58 μM ProddIP were incubated with pancreatin and analysed by the LC–MS/MS method (data not shown). The variation of ProddINP concentrations and peak areas corresponding to diglyceryl and the 2-monoglyceryl-metabolites in the lipolysis medium were expressed as a function of incubation time (Fig. 6d–f).

When the incubation time increased, ProddIP concentration in the lipolysis medium decreased. Unlike ProddINP, the addition of pancreatin medium to ProddIP loaded liposomes led to an instantaneous loss of 90% of ProddIP initial concentration in the lipolysis mixture (Fig. 6d). After 5 min of enzymatic incubation, only 5% of ProddIP initial amount was detected. Impressively, the lipolysis of ProddIP seemed to be completed after 10 min incubation, since no traces of ProddIP were detected under our experimental conditions after this time. Likewise, the relative transformation rate of ProddIP was estimated around 11 μM/min.

As a function of time of incubation of ProddIP with pancreatin, the diglyceryl and the 2-monoglyceryl-metabolites appeared (Fig. 6e and f, respectively). The diglyceryl-ProddIP metabolite appeared immediately at the beginning of incubation (0.5 min) and progressively disappeared; only traces remained detectable after 15 min of incubation (Fig. 6e). The monoglyceryl-ProddIP metabolite rapidly appeared in the medium and reached a step after 5 min of incubation.

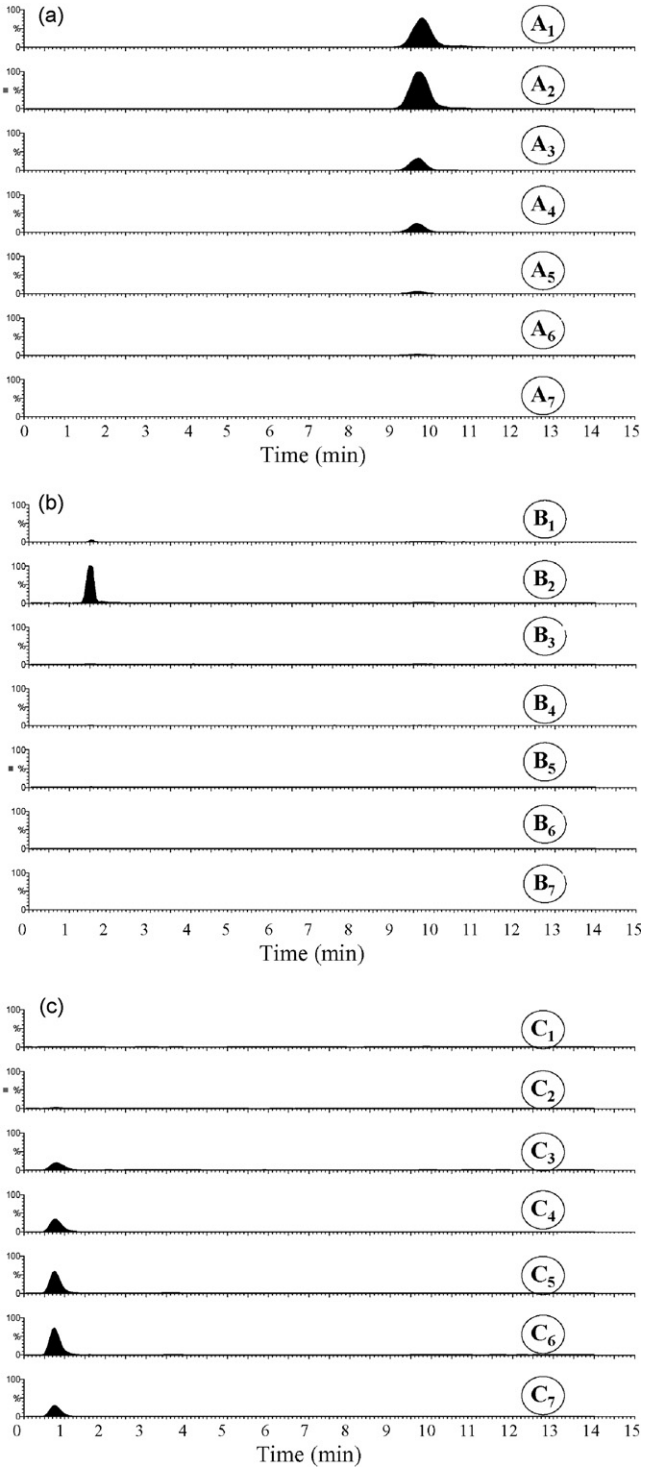

Fig. 5. Mass chromatograms of (a) ProddINP, (b) diglyceryl-ProddINP, (c) 2-monoglyceryl-ProddINP: mass chromatograms of ProddINP in liposomes (A1), ProddINP extracted from lipolysis medium after 0.5 min (A2), 5 min (A3), 10 min (A4), 15 min (A5), 30 min (A6), 60 min (A7) of incubation with pancreatin. Mass chromatograms of the diglyceryl-ProddINP in liposomes (B1), extracted after 0.5 min (B2), 5 min (B3), 10 min (B4), 15 min (B5), 30 min (B6), 60 min (B7) of incubation with pancreatin. Mass chromatograms of the 2-monoglyceryl-ProddINP in liposomes (C1), extracted after 0.5 min (C2), 5 min (C3), 10 min (C4), 15 min (C5), 30 min (C6), 60 min (C7) of incubation with pancreatin.

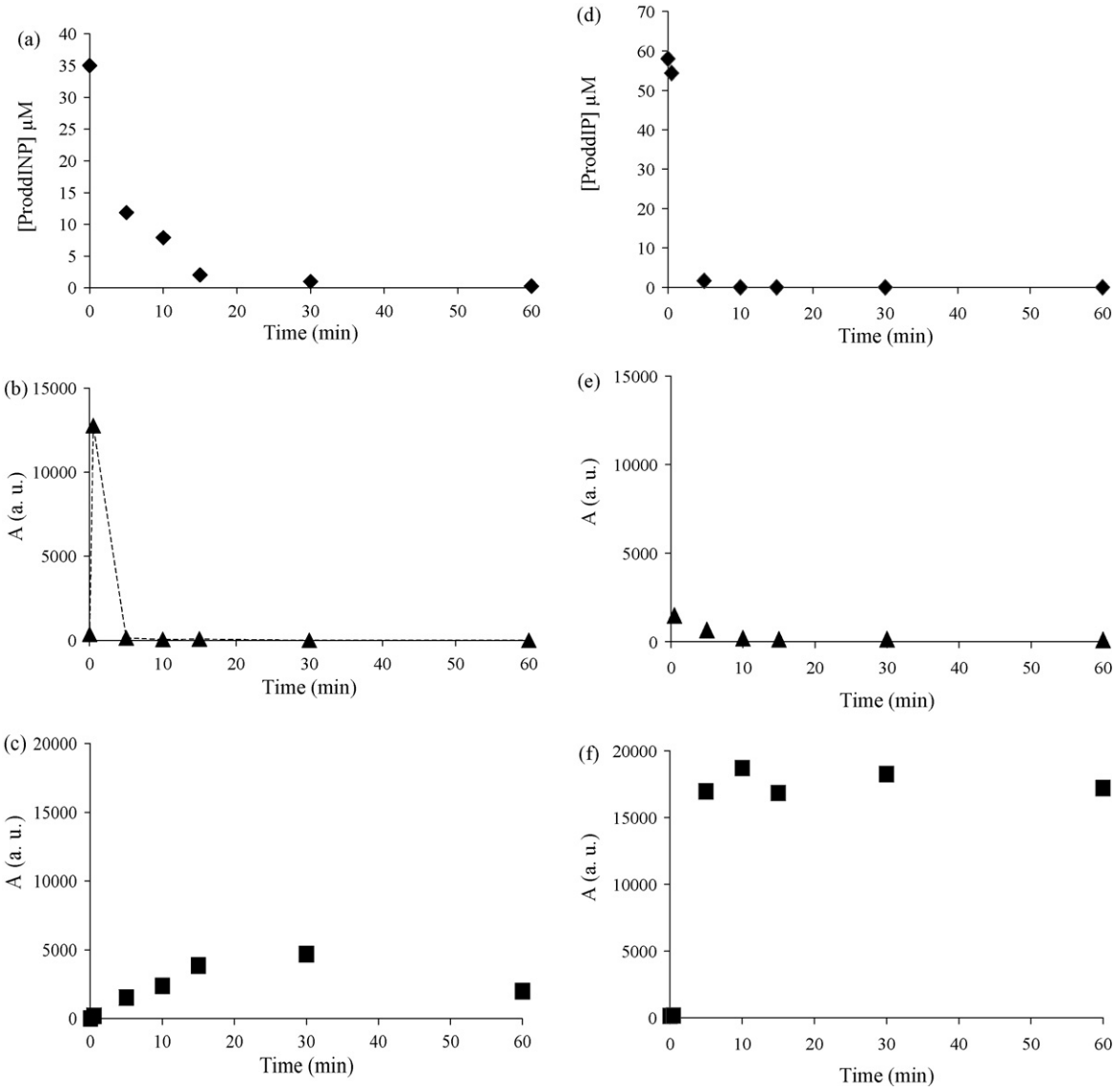

Fig. 6. Biotransformation of liposomal suspensions of 35 µM of ProddINP and 58 µM of ProddIP and their di- and 2-monoglyceryl-metabolites as a function of incubation time with pancreatin: (a) ProddINP concentration, peak areas of (b) diglyceryl-ProddINP and (c) monoglyceryl-ProddINP, (d) ProddIP concentration, peak areas of (e) diglyceryl-ProddIP and (f) 2-monoglycéril-ProddIP in lipolysis mixture.

3.2. Metabolism of didanosine prodrugs during incubation with HIV-1 infected cells

Didanosine, ProddIP and ProddINP loaded liposomes were incubated during 24 h with PBMC post-infected by HIV-1. Intracellular concentrations of ddA-TP were determined using LC–MS/MS. After incubation with 1 µM of ddI, ProddIP or ProddINP, only ddA-TP arising from ddI was detected. After incubation with 10 and 20 µM of ddI, ProddIP or ProddINP, only ddA-TP arising from ddI or ProddINP was detected with a slightly decreased concentration for ProddINP. It appeared that the biotransformation of ProddIP led after 24 h to a concentration of ddA-TP below the LOQ value of 25 fmol.

4. Discussion

The biomimetism approach of our glycerolipid prodrugs has been studied at two steps: the metabolism of prodrugs by enzymes of gastrointestinal (GI) tract and of their metabolites by intracellular kinases into HIV-1 infected cells.

4.1. Metabolism of prodrugs by gastrointestinal enzymes

4.1.1. Use of a LC–MS/MS method

The study of lipidic prodrug metabolism by gastrointestinal enzymes was achieved thanks to a LC–MS/MS method. Unlike other methods following only disappearance of prodrug, this technique allowed us to identify, separate and detect simultaneously the initial prodrugs and their metabolites in a complex lipidic medium.

ProddINP and ProddIP could be detected by different molecular masses and specific MRM transitions. Nevertheless, they shared specific fragmentation pathways (Fig. 3). The first fragmentation route, which corresponded to the cleavage of the glycosidic bond and thus, the loss of hypoxanthine (M = 136; Fig. 3), produced the daughter ions m/z 765.5 and 845.4 observed for ProddINP and ProddIP mass spectra, respectively (Fig. 3). The loss of 136 was observed by Huang et al. for ddI fragmentation (Huang et al., 2004). Noteworthy, the most abundant protonated ion a′ (m/z 137) of hypoxanthine, produced by the cleavage of the parent ion, was only observed during the fragmentation of ProddINP. Another fragment ion was detected at m/z 551.4, which was a result of the same

fragmentation pattern of both prodrugs (Fig. 3, ion b). This observed ion corresponded to the cleavage at position *sn*-2 of the triacylglyceryl prodrug. Noteworthy, the complementary ions produced by this fragmentation route, ions b′, were not observed. Further fragmentations of the ions *m*/*z* 845.4 and 765.5 have confirmed this pathway (data not shown). The study of fragmentation pathways of prodrugs allowed determining the specific transitions that were used to identify the metabolites in the lipolysis medium. Only the expected monoglyceryl- and diglyceryl-metabolites were detected during the incubation of each ddI prodrug with pancreatin. The fragmentation pathway of these molecules evidenced that they shared the same loss of 136 mass and the formation of the daughter ion *m*/*z* 137.0 (Fig. 4). The specific MRM transitions were chosen to follow enzymatic reaction by LC–MS/MS analyses. Finally this method allowed evaluating the relative biotransformation rate of the studied prodrugs.

4.1.2. Comparison of didanosine prodrugs biotransformations using a lipolysis model

Lipid digestion models are widely used as *in vitro* tools to evaluate lipid-based drug delivery systems (Porter and Charman, 2001; Porter et al., 2007). We chose to use pancreatin which is present in the artificial intestinal medium described in European Pharmacopea 6th ed. Numerous authors have evidenced the need to design biorelevant mediums to evaluate the release and metabolism of prodrug-loaded lipid formulations *in vitro* (Porter et al., 2007; Jantratid et al., 2008). Pancreatin contains various enzymes like lipases and phospholipases. Because pancreatic lipase needs the presence of co-lipase and bile salts to adsorb at the surface of lipid droplets and phospholipase A2 the presence of Ca^{2+} ions and bile salts to be active, we completed this medium with calcium ions, lecithin and a bile salt (sodium taurocholate, TC) according to the model described by MacGregor et al. (1997). A TC concentration of 20 mM was used to mimic fed conditions. The molar rate between bile salt and lecithin was 4:1 as encountered in FaSSIF, FeSSIF and other modified mediums (Porter et al., 2007; Jantratid et al., 2008). In these conditions, the excess of sodium taurocholate (TC/lipid ratio > 1) allowed the complete solubilisation of DPPC and EPC vesicles and the formation of mixed micelles (Andrieux et al., 2004, 2009) able to dissolve lipid and prodrug metabolites in lipolysis medium. The solubility of prodrugs and their metabolites into the gastrointestinal tract is of great importance to increase their intestinal absorption.

After incubation with the chosen medium at different times, extraction of prodrugs and metabolites by a validated protocol, we determined by an optimised LC–MS/MS method the relative biotransformation rate of our glycerolipidic prodrugs. The lipolysis of both prodrugs was practically completed after 30 min of enzymatic incubation (Fig. 6a and d). These results demonstrated that the synthesized prodrugs of didanosine were recognized by the enzymes of pancreatin as glycerolipids due to their structural homology with triglycerides. The metabolism process evidenced the formation of two major metabolites which were identified as the 2-monoglyceryl-ProddINP and the 2-monoglyceryil-ProddIP at the end of the digestion (Fig. 6c and f). The rapid appearance and then disappearance of diglyceryl-metabolites suggested that they were intermediary metabolites (Fig. 6b and e). The expected metabolism pathway of our glycerolipidic prodrug described in Fig. 2 and the biomimetism of our strategy was then confirmed.

As a matter of fact, ProddIP rate of lipolysis was twice higher than that of ProddINP. The implication of phospholipases in the metabolism of ProddIP was not excluded. The phosphate group in ProddIP induced a structural homology with phospholipids and could lead to its metabolism by pancreatic phospholipases (Rogalska et al., 1990; Ransac et al., 1990). Phospholipase A2 metabolizes phospholipids in phospho-diglycerides. This might explain the increased rate of ProddIP biotransformation when compared to ProddINP. The difference between the spacers used in both prodrugs could also influence their respective enzymatic degradation but in both cases the complete metabolism was obtained in 30 min which is compatible with *in vivo* digestion. Moreover this result suggested that the difference of size between both formulations (187 ± 75 and 777 ± 90 nm for ProddINP and ProddIP formulations, respectively) has no influence on the enzymatic activity of lipases at the surface of liposomes in the experimental conditions.

4.2. Biotransformation of prodrugs metabolites by intracellular kinases

After their metabolism into the GI tract, the monoglyceryl-metabolites of our prodrugs, would be transformed or not in new triglycerides by reesterification with fatty acids in enterocytes but associated into chylomicrons and then excreted into mesenteric lymph from where they can reach the systemic circulation via the thoracic lymph duct as expected from triglycerides metabolism (Drusano et al., 1992; Porter and Charman, 1997; Lambert, 2000). From systemic circulation, these neo-synthesized triglyceride prodrugs carried into lipoproteins could be distributed into the body and active on HIV infected cells. Didanosine may be released by prodrug degradation because the spacer chosen can be cleaved by esterases. This release could occur in the blood or in the cells after penetration of the more hydrophobic prodrug. It is well known that the intracellular didanosine needs to be sequentially phosphorylated by host cell kinases to the 5′-triphosphate derivative, ddA-TP to inhibit the viral reverse transcriptase (Fig. 1). In order to study *in vitro* this ultimate metabolism step, ProddIP and ProddINP loaded in liposomes have been incubated during 24 h with PBMC post-infected by HIV-1 and intracellular concentrations of ddA-TP were determined using a previously optimised LC–MS/MS method (Becher et al., 2002). Initial prodrugs and DPPC liposomes were used as a first approach to mimic neo-synthesized glycerolipidic prodrugs and lipoproteins, respectively. The results showed concentrations slightly decreased of ddA-TP arising from ProddINP compared to ddI and concentrations below 25 fmol from ProddIP. First, the detection of intracellular ddA-TP evidenced that ddI has been released from prodrug after a likely enzymatic degradation. Moreover these results confirmed the antiviral activities previously measured in these cells: ED50 = 1.2, 2.8 and 28.5 μM of ddI, ProddINP and ProddIP, respectively (Lalanne et al., 2007a). The difference between antiviral activities and intracellular metabolism in ddA-TP of both prodrugs can be attributed to a lower penetration of the phosphorylated prodrug, ProddIP than the non-phosphorylated one, ProddINP. The charge of the phosphate-ddI was unfavourable for cell penetration as observed in other studies (Bonnafe et al., 1996). This point suggested that the release of ddI or phosphate-ddI from prodrugs occurred in the extracellular medium before the penetration into the cells.

5. Conclusion

The didanosine glycerolipidic prodrugs behaved as glycerolipids since they were metabolised after incubation with pancreatic lipases. The chosen medium seemed to be convenient in order to mimic the digestion medium that could be encountered by our prodrugs incorporated into liposomes after their oral administration. In addition, a significant difference in the relative biotransformation rates of both prodrugs was observed. Under our experimental conditions, ProddIP was metabolised twice more rapidly than ProddINP suggesting an implication of some phospholipases in ProddIP degradation. The 2-monoglyceryl-ProddIP and the 2-monoglyceryl-ProddINP were the main observed metabolites after

30 min of lipolysis. Moreover, the detection of ddA-TP into HIV-1 infected cells after their incubation with ProddINP mixed liposomes evidenced that they can release ddI which can penetrate into the cells before metabolism by intracellular kinases and that the lipid conjugation did not suppress the pharmacological activity of didanosine. The intracellular concentration of ddA-TP arising from ProddIP was too low to be detected in the experimental conditions. It was to note that the increased biotransformation rate of ProddIP by pancreatic enzymes could lead *in vivo* to an increased blood concentration of its metabolite and could compensate its lower penetration into infected cells.

These results confirmed that the synthesized glycerolipidic prodrugs of didanosine could be investigated for a biomimetic approach with final aiming of increasing the drug oral bioavailability by enhancing intestinal absorption. Further experiments are ongoing in order to evaluate and compare the uptake of these prodrugs by CaCo-2 cell lines after their metabolism by pancreatin lipases.

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