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Liposomal formulation of a glycerolipidic prodrug for lymphatic delivery of didanosine via oral route

M. Lalanne [a], K. Andrieux [a,*], A. Paci [b], M. Besnard [a], M. Ré [b], C. Bourgaux [a], M. Ollivon [a], D. Desmaele [c], P. Couvreur [a]

[a] *Univ. Paris-Sud, UMR CNRS 8612, IFR 141, F-92296 Châtenay-Malabry, France*
[b] *Univ. Paris-Sud, UPRES EA 3535, IFR 54, Institut Gustave Roussy, F-94805 Villejuif, France*
[c] *Univ. Paris-Sud, UMR CNRS 8076, IFR 141, F-92296 Châtenay-Malabry, France*

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Abstract

Didanosine is a polar drug with poor membrane absorption and high hepatic first pass metabolism. This study aimed at developing a lipidic formulation of a glycerolipidic prodrug of didanosine in order to improve its bioavailability. In the course of a preformulation study, the glycerolipidic prodrug of didanosine was characterized by microscopy, DSC and XRDT. In anhydrous conditions, the prodrug displayed a polymorphic behaviour similar to that of triglycerides. Then, we evaluated three types of lipidic formulations (emulsions, mixed micelles and liposomes) in order to encapsulate the prodrug. Solubilities in water – even in the presence of taurocholate micelles – but also in some oils were very low (max 244 μg/mL) as the prodrug was found to be amphiphilic (log $P = 2$). On the contrary, the prodrug was found to be perfectly incorporated in dipalmitoylphosphatidylcholine (DPPC) multilamellar liposomes up to a ratio of 1:5 (mol:mol) prodrug:DPPC as suggested by HPLC-UV and DSC experiments. Moreover, these liposomes could be freeze-dried whereas the chemical integrity of the prodrug was preserved. Then, the freeze-dried liposomal preparation could be formulated as gastro-resistant capsules to prevent didanosine from acidic degradation. Further experiments are on the way to evaluate *in vitro* the absorption of prodrug incorporated in liposomes by enterocytes.

Keywords: Glycerolipidic prodrug; DSC; XRDT; Liposomes; Didanosine

1. Introduction

Didanosine (ddI) is a reverse transcriptase inhibitor, well-known for the treatment of HIV infections. Administered orally, ddI has a poor bioavailability (20–40%) (Balimane and Sinko, 1999). This is attributed to the acidic degradation of ddI in the stomach, to its poor absorption due to the hydrophilic character of this molecule and to the hepatic first pass metabolism. The design of glycerolipidic prodrugs is a strategy which was previously proposed to enhance the oral bioavailability of certain compounds by mimicking long chain triglycerides (Porter and Charman, 1997; Charman and Porter, 1996). Physiologically, 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 can reach the systemic circulation via the thoracic lymph duct, thus, by-passing the liver (Lambert, 2000; Porter and Charman, 1997). Thus, we have considered the synthesis of a glycerolipidic prodrug of didanosine in order to increase enterocyte absorption and lymphatic transport and to by-pass the hepatic first pass metabolism, hence leading to increase the bioavailability of the drug. Noteworthy, ddI needs to be sequentially phosphorylated by host cell kinases to the 5′-triphosphate derivative to be active. The first phosphorylation is, however, the limiting step (Tan et al., 1999). Thus, another approach to improve ddI activity is to by-pass the first cellular phosphorylation by synthesizing a glycerolipidic prodrug which is mono-phosphorylated (ProddIP) (Fig. 1). In a previous paper (Lalanne et al., 2007), we described the synthesis of this prodrug of ddI. The purity was

* Corresponding author. Tel.: +33 146835909; fax: +33 146619334.
E-mail addresses: karine.andrieux@u-psud.fr, karine.andrieux@cep.u-psud.fr (K. Andrieux).

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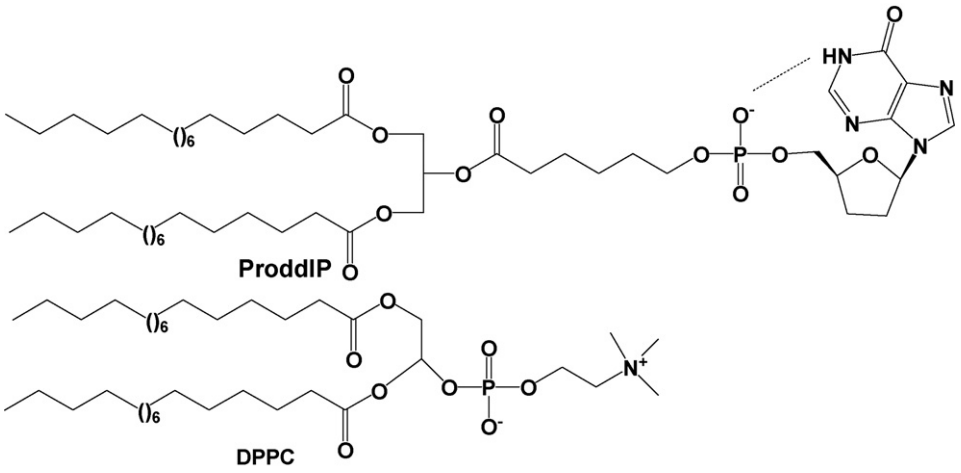


Fig. 1. ProddIP and DPPC structures.

evidenced by a validated HPLC-UV dosage method and mass spectrometry experiments.

Three types of formulations are generally suggested to enhance lymphatic absorption: emulsions, lipid mixed micelles and liposomes (Gershanik et al., 2000; Gershanik and Benita, 2000; Gursoy and Benita, 2004; Hauss et al., 1998). Because of their rich lipidic composition, these formulations generally increase chylomicron synthesis by the enterocytes. Lipophilic drugs can, indeed, gain access to the intestinal lymph through association with the products of lipid digestion and can be secreted in association with lymph lipoproteins. That is why it is also important to associate lipophilic prodrugs to lipidic compounds (long chain triglycerides or long chain phospholipids) into the formulation.

Thus, the aim of this study was to develop a lipidic formulation of this new glycerolipidic prodrug of monophosphate didanosine for oral administration. A preformulation study was first performed by polarized light microscopy, differential scanning calorimetry (DSC), and X-ray diffraction (as a function of temperature) (XRDT). Then, the ability of the three above mentioned formulations (emulsions, mixed micelles and liposomes) to incorporate the prodrug has been investigated by HPLC-UV, DSC and X-ray diffraction measurements. The stability and the protection against acidity of didanosine in the lipidic formulation chosen were also investigated.

2. Material and methods

2.1. Products

All the chemical reagents were obtained from Sigma–Aldrich (St. Louis, USA) except the ddI which was obtained from Bristol Myers Squibb (NY, USA). The solvents for the analytical section were obtained from Carlo Erba (Rodena, Italy).

Almond oil and soya oil were obtained from Cooper (Melun, France) and fish liver oil from SIRH (Fecamp, France). sodium taurocholate, sodium chloride and DPPC were obtained from Sigma (St. Louis, USA). Na_2HPO_4 and NaH_2PO_4 were obtained from VWR Prolabo (Fontenay sous Bois, France).

The prodrug (ProddIP) was synthesized as described in a previous study (Lalanne et al., 2007).

2.2. Polarized light microscopy

The prodrug and its formulation were observed by microscopy between crossed polarizers and with a λ/4 retarder in white light using a Nikon E600 Eclipse direct microscope (Champigny/Marne, France) equipped with a long focus objective (LWD 40 × 0.55; 0–2mm). NIKON Coopix 950 camera was used as a picture recorder with a resolution of 1600 × 1200 pixels.

2.3. DSC experiments

Thermal analyses were conducted by DSC, using a DSC-7 (Perkin-Elmer, St. Quentin en Yvelines, France). Samples were loaded in aluminium pans of 40 μL (pan, part no. BO14-3021, and cover, part no. BO14-3004) hermetically sealed. An empty, hermetically sealed aluminium pan was used as reference. Calibration was performed with indium (mp 156.60 °C, $\Delta H_m = 28.45$ J/g) and *n*-decane (mp −29.66 °C). Melting behaviour of the prodrug was monitored with the temperature scanning program set from 10 to 60 °C at a rate between 1 and 20 °C/min and crystallization behaviour was monitored from 60 to 10 °C at the same rate. For the thermal treatment, the prodrug was heated 15 min at 36 °C, quickly cooled to 10 °C and melting behaviour was then monitored from 10 to 60 °C at 10 °C/min.

2.4. XRDT measurements

X-ray experiments were performed with a fine-focus Cu anode source; Cu Kα (λ = 1.54 A) radiation was selected and line focused by a multilayer mirror and collimated by slits. A microcalorimeter cell, MICROCALIX (Keller et al., 1998; Ollivon et al., 2006), which allowed simultaneous thermal and X-ray measurements, was used as sample holder. Samples were loaded in Lindemann glass capillaries (diameter 1.5 mm) and

heated from 10 to 60 °C at 0.1 °C/min. Small-angle (SAXS) and wide-angle (WAXS) X-ray scattering patterns were recorded simultaneously using two position-sensitive linear gaz detectors; the scattered intensity was reported as a function of the scattering vector $q = 4p \sin\theta/\lambda$ where θ is half the scattering angle and λ is the wavelength. The calibration of the detectors was made with the crystalline β form of high-purity tristearin and with silver behenate, as previously described (Brubach et al., 2004).

2.5. HPLC-UV experiments

The HPLC-UV apparatus consisted of a Nucleosil® C18 column, 250 mm, 10 μm (Hypersil, Cheshire, Great Britain), a Waters 717 plus Autosampler® (Waters, St. Quentin en Yvelines, France), an isocratic pump (Waters model 510®) and a UV detector (Waters 486®). Data were treated with Millenium® software (Waters, St. Quentin en Yvelines, France). The absorption maximum was found to be 280 nm. The limit of detection (LOD) was 1.2 ng/μL and limit of quantification (LOQ) 3.7 ng/μL as previously described (Lalanne et al., 2007).

Concerning the prodrug solubility studies in oil, water and micellar solution of 20 mM sodium taurocholate, 1 mg of prodrug was mixed with 200 μL of the solvent (oils, water or micellar solution). The mixture was then ultrasonicated for 15 min and then kept under mechanical stirring at 4 °C during 48 h before centrifugation for 15 min at room temperature and 15,000 × *g* (Bioblock Scientific 112®, Centrifuge, Sigma, Steinheim, Germany). The pellet was then dissolved in 1 mL of ethyl acetate and diluted 10 times in methanol. The supernatant was diluted 10 times in ethyl acetate. Ten microlitres of each mixture was injected in the HPLC-UV apparatus.

The log *P* evaluation was inspired from log *P* evaluation previously described (Shah et al., 1994; Constantinides et al., 1994). Briefly, equal amount (200 μL) of water and octanol were ultrasonicated for 1 h in the presence of 1 mg of ProddIP at 37 °C. The mixture was then centrifuged at 15,000 × *g* for 30 min (Bioblock Scientific®, Sigma, Steinheim, Germany). The organic phase was diluted five times in methanol before being injected in the HPLC-UV chromatographic system, while the aqueous phase was directly injected in the HPLC-UV apparatus. The log *P* was calculated according to the following formulae:

$$\log P = \log\left(\frac{[\text{prodrug}]_{\text{octanol}}}{[\text{prodrug}]_{\text{water}}}\right)$$

where $[\text{prodrug}]_{\text{octanol}}$ and $[\text{prodrug}]_{\text{water}}$ were the concentrations of the prodrug as determined in the octanol and water phases, respectively.

2.6. Preparation of ProddIP/DPPC mixtures

The mixtures of ProddIP and dipalmitoylphosphatidylcholine (DPPC) at various ratios (0:1, 1:10, 1:6, 1:5; 1:3, 1:2, 1:1, 2:1, 5:1, 10:1 (mol:mol)) were prepared by mixing adequate quantities of ProddIP and DPPC in 0.2 mL of methanol and 0.1 mL of chloroform. The organic solvents were the evaporated under vacuum using a rotavapor, and the residual traces of organic solvents were evaporated under low vacuum. The lipidic film was then rehydrated with water with a 10:90 (w/w) lipid:water ratio. These mixtures were analyzed by DSC during their heating between 10 and 60 °C at 10 °C/min.

2.7. Preparation and entrapment efficiency evaluation of DPPC liposomes

The multilamellar vesicles (MLV) of DPPC were prepared according to Bangham's method (Bangham et al., 1974). Briefly, the prodrug of ddI (0.4 mg) and DPPC (1.5 mg) were dissolved in chloroform (0.1 mL) and methanol (0.2 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 lipidic 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 with 0.8 μm filter (Sartorius®, Palaiseau, France), three times. The same procedure was applied to prepare DPPC MLV.

The entrapment efficiency was evaluated by HPLC-UV. The liposome suspension was centrifuged at 15,000 × *g* during 15 min (Bioblock Scientific 112®, Centrifuge, Sigma, Steinheim, Germany) at room temperature. The pellet was diluted 10 times in methanol before being injected in the HPLC-UV chromatographic system. The supernatant was directly injected in the HPLC-UV apparatus.

2.8. Liposome size measurement and stability at 4 °C

The mean diameter of the prepared liposomes was determined by quasielastic laser light scattering with a Nanosizer Coulter model N4 MD® (Coulter Electronic, Margency, France). The measurement was done 15 min after liposome preparation in order to have a steady state. Size evolution was investigated during 1 week to 2 months. The samples were kept at 4 °C during the stability study to keep the glycerolipidic prodrug away from the degradation. Measurements were done at room temperature. Unimodal and Size Distribution Processor (SDP) results were registered. When the population was multimodal, the mean diameter of each population and their standard deviation (S.D.) were given according to the SDP results with the corresponding percent of intensity light scattering (presented as amount (%) in Tables 2 and 4).

2.9. Zeta potential experiments

Zeta potentials of ProddIP:DPPC mixed liposomes (1:5 (mol:mol)), and DPPC liposomes were measured using a Zetasier Nano Series® (Malvern Instruments, Malvern, United Kingdom). For zeta potential determination, liposomal suspensions were diluted 10 times in MilliQ® water (Millipore, St. Quentin en Yveline, France).

2.10. Degradation in acidic conditions

One milliliter of the ProddIP:DPPC liposomes 1:5 (mol:mol) suspension (in phosphate buffer) was mixed together with 2 mL of a HCl 1N solution in order to have a final pH around 1. This mixture was kept for 30 or 60 min to mimic stomach conditions (Silbernagl and Despopoulos, 2001; Mion and Roman, 2003). The mixture was then neutralized with a NaOH 1N solution. A control experiment was performed in the same conditions using 1 mg of pure ddI. The samples were then analyzed with the HPLC-UV according to the procedure described before.

3. Results

3.1. Characterization of ProddIP

As a prerequisite for its formulation, the new ddI prodrug was first characterized by polarized light microscopy (PLM), differential scanning calorimetry (DSC) and X-ray diffraction as a function of temperature (XRDT).

The PLM pictures of the prodrug taken at room temperature showed that this compound was crystallized (Fig. 2). The ddI prodrug was then analyzed by DSC between 10 and 60 °C at different heating rates (1, 5, 10 and 20 °C/min, data not shown); it exhibited complex pattern with increasing number of thermal events when the heating rate was decreased. The DSC curve recorded during the heating of the prodrug at 1 °C/min (Fig. 3(a) top) showed a double endothermal event at 27.5 °C followed by an exothermal event at 36 °C and two endotherms at 37.8 and 44.2 °C. During the following cooling at 1 °C/min of the same sample, the DSC recording exhibited only two exothermal events at 33 and 33.8 °C (Fig. 3(a) bottom) which seemed to correspond to the crystallization of the two forms observed at low temperature. A thermal treatment was applied to the ProddIP (Fig. 4). A sample of ProddIP was heated from 10 to 36 °C, and after a pause of 15 min, frozen down to 10 °C. The heating of this sample was finally recorded at 10 °C/min and compared with the recording at the same rate without this procedure. Thanks to the thermal treatment, two new endothermal events could be observed at 39.2

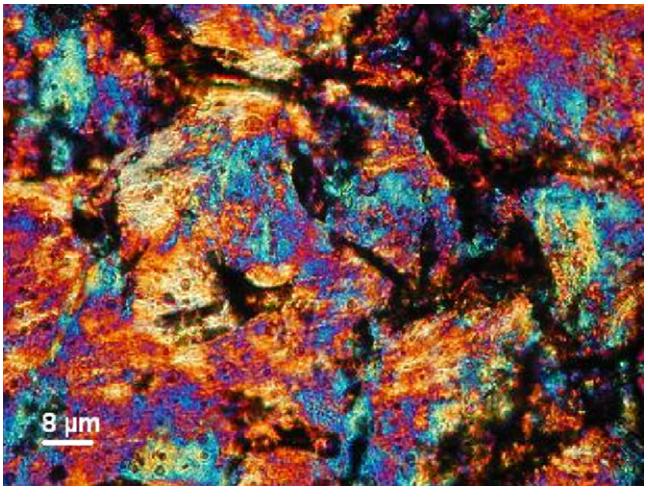


Fig. 2. Crystals of ProddIP in polarized light microscopy.

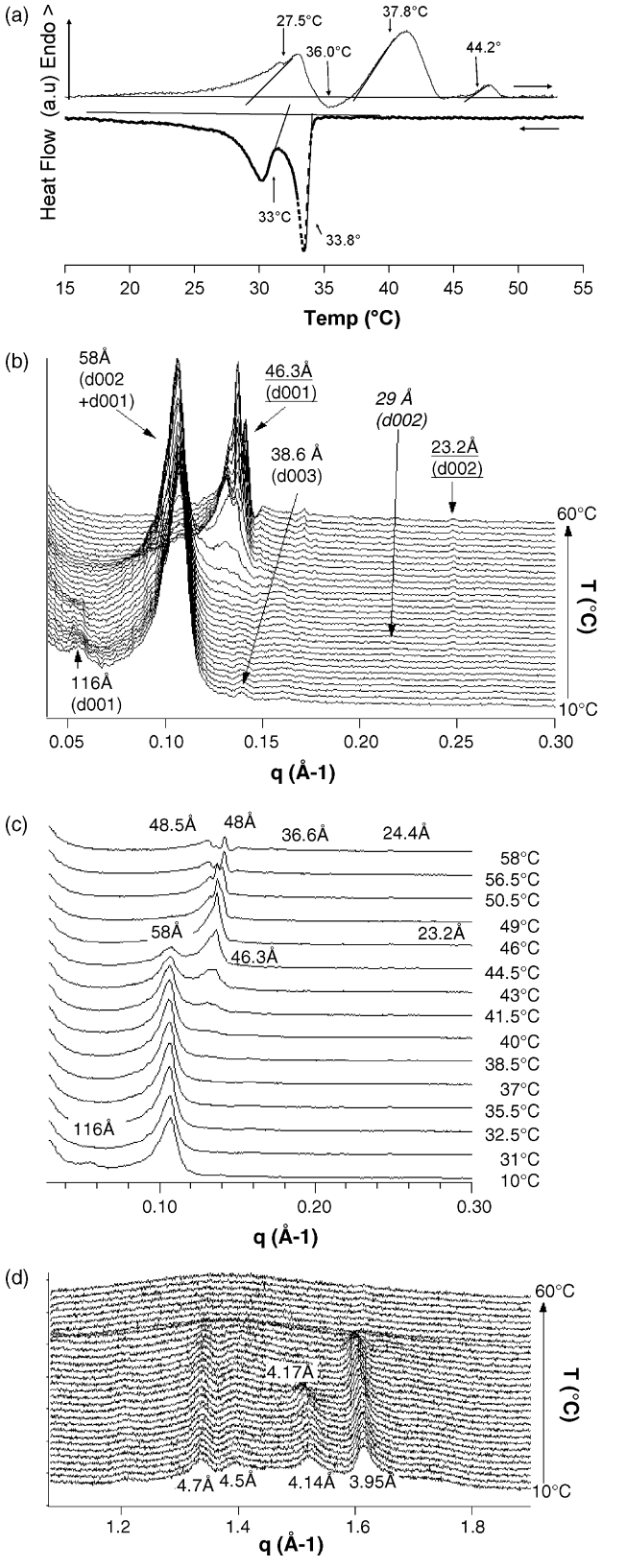


Fig. 3. Characterization of thermal and structural properties of ProddIP at $10 \leq T \leq 60$ °C. (a) DSC recordings obtained by heating followed by cooling at 1 °C/min, (b)–(c) SAXS and (d) WAXS recorded as a function of temperature on heating at 0.1 °C/min showing phase transition and multiple melting.

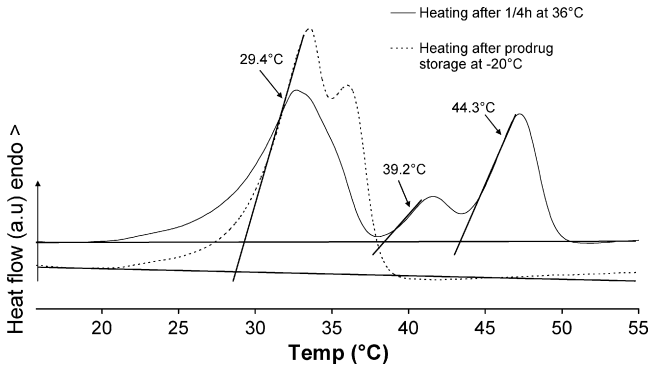


Fig. 4. DSC recordings of ProddIP, before and after thermal treatment (1/4 h at 36 °C) obtained at 10 °C/min.

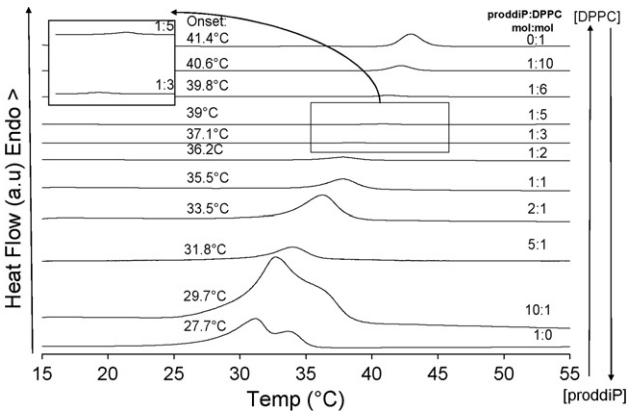


Fig. 5. Influence of ProddIP insertion on DPPC thermal properties.

and 44.3 °C and the double endotherm at 29.4 °C was replaced by only one endotherm with a lower enthalpy. This suggests that the pause at 36 °C followed by the freezing allowed modifying the supramolecular organization of the prodrug. These DSC results suggested that the prodrug was organized in different polymorphic forms which existed according to a specific temperature range. These results were confirmed by recording X-ray diffraction diagrams (SAXS and WAXS) during the heating of the samples at 0.1 °C/min (Fig. 3b–d). The prodrug appeared to be organised in several (principally 2) crystalline lamellar phases at lower temperature. One of the phases had a repeat distance of 116 Å. This phase disappeared at 32.5 °C while the main phase, with a repeat distance of 58 Å did between 43 and 49 °C. New lamellar phases (perhaps 4) appeared during the heating of the prodrug above 36 °C. The main phase at higher temperature had a repeat distance of 46.3 Å and coexisted until 58 °C with a progressive decrease in the intensity of the peak.

At the same time, the solubility of the ddI prodrug was investigated in different aqueous solutions or oils by chromatographic determinations (Table 1) according to the previously validated analytical method (Lalanne et al., 2007). The solubility of the prodrug appeared to be influenced by the pH of the aqueous solution: from pH 8 to pH 4, the solubility was increased (2×) but still remained very low (25 μg/mL). In a 20 mM sodium taurocholate (TC) micellar solution, the solubility was increased up to 108 μg/mL whereas it reached 143.1 μg/mL in the almond oil. Moreover, the log *P* was found to be around two indicating that the prodrug may be classified as an amphiphilic compound (Gulati et al., 1998).

Table 1
Solubility of ProddIP in different solvents as determined by HPLC-UV analysis

	Solubility (μg/mL)
Water (pH 8)	10.5
Water (pH 4)	25.3
Taurocholate micelles	108
Soya oil	36.8
Liver fish oil	132.7
Almond oil	143.1

3.2. Encapsulation of the ddI prodrug into DPPC liposomes

Considering the results of preformulation, the encapsulation of ddI prodrug into DPPC liposomes was evaluated by complementary experiments of DSC, HPLC dosage and microscopy.

First, multilamellae of hydrated DPPC with increasing ratio of the prodrug were analyzed by DSC in order to evaluate their capacity to incorporate ProddIP in the bilayers (Fig. 5). The DSC recording for pure DPPC shows the widely reported features of the pure phospholipid (Morrow and Davis, 1988; Vist and Davis, 1990; McMullen et al., 1993; Small, 1986): a sharp chain-melting transition occurring at 41.4 °C. Increasing the proportion of ProddIP led to a significant decrease of the onset temperature and of the melting enthalpy of the main chain-melting transition until a ProddIP:DPPC ratio of 1:3 (mol:mol). With higher amount of ProddIP, a melting endotherm was again observed. These data suggested that the optimal ratio allowing insertion of the ProddIP into DPPC liposomes – without their destabilization – was 1:5 (mol:mol). Therefore, further prodrug-loaded DPPC liposomes were prepared at this ratio. The sizes of the obtained MLV were relatively high (Table 3), but decreased with successive filtrations (three time on 0.8 μm filter). Two populations mean size of which is ≤1 μm were then observed. The insertion of ProddIP into DPPC liposomes was further determined by HPLC dosage in the pellet after centrifugation of the prodrug-loaded liposomes showing a very good insertion of ProddIP into liposomes before and after filtration (>80%) (Table 2). The amount of ProddIP in the supernatant could not be established because the concentration found was below LOQ (3.7 ng/μL).

Microscopic observation of the 1:5 (mol:mol) prodrug-loaded DPPC liposomes sample showed the absence of ProddIP crystals, suggesting that the prodrug was completely incorporated into the liposomes. These liposomes have been stored during a month at 4 °C without any change of their size. Zeta potential was −57 ± 7 mV.

While it was observed that the degradation of ddI was increased – comparatively to ddI free in water suspension – when the prodrug was incorporated into liposomes (Table 3), liposomes were freeze-dried in order to allow their formulation as gastro resistant capsules. Assays were performed with dif-

Table 2
Sizes of MLV and entrapment efficiency (%) of ProddIP into ProddIP:DPPC 1:5 (mol:mol) liposomes before and after filtration (three times at 0.8 μm)

	Population	Amount (%)	Size (nm)	S.D. (nm)	Entrapment efficiency (%)
Before filtration	1	8	123	50	93
	2	92	2035	540	
After filtration	1	37	156	21	83
	2	63	777	90	

Table 3
Degradation (%) of ddI and ProddIP in acidic medium

Degradation of	After 30 min at pH 1	After 60 min at pH 1
ddI	36.4	54.5
ProddIP	55.8	81.7

ferent amounts of sucrose as cryoprotectant (Table 4). For the 1:10 lipid:sucrose ratio (w/w), the size of the two populations of liposomes was kept unchanged after rehydratation as well as the encapsulated amount of ProddIP (>80%).

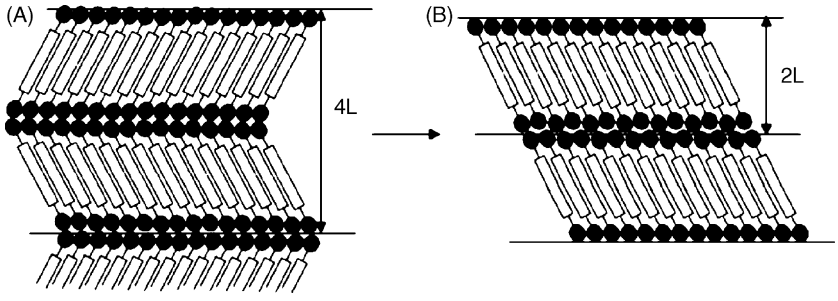
4. Discussion

With the aim of improving the bioavailability of ddI after oral administration, we first evaluated the supramolecular organization of a newly synthesized diglyceridic prodrug of ddI by comparing with that of triglycerides.

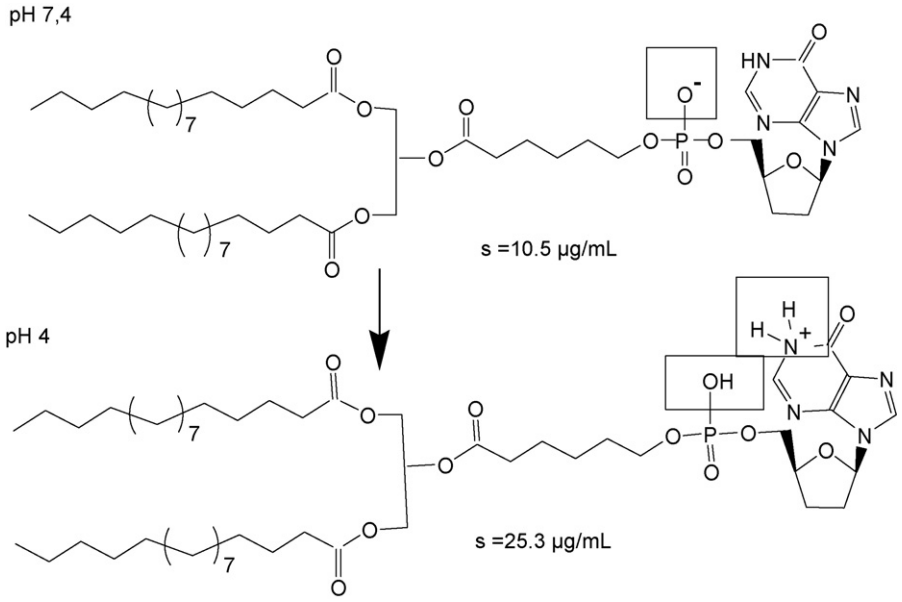
The DSC experiment suggested that the prodrug was organised, at low temperature, in various polymorphic forms which melted with increasing temperature. Then it recrystallized in new polymorphic forms melting at high temperature. It is hypothesized that, at low temperature, the observed polymorphic forms were metastable whereas slow heating rate allowed the recrystallization in new polymorphic forms. In order to explore this hypothesis, a thermal treatment was applied to ProddIP sample with a pause at 36 °C which corresponded to the recrystallization temperature. This experiment confirmed the reorganization of the prodrug in more stable polymorphic forms. An increase of the pause at 36 °C would improve the organization of ProddIP in more stable polymorphic phases which would be more easily characterized in further experiments. The polymorphism of ProddIP was further evidenced by XRDT experiments. The identification of the different polymorphic phases was difficult because of the complexity of the prodrug organization. Numerous phases were observed during heating which allowed a complete reorganization of the prodrug. All these results suggested that ProddIP was packed in a supramolecular organization close to the one of the triglycerides (Lopez et al., 2000, 2001, 2002; Michalski et al., 2004). At 10 °C, one phase with a repeat distance of 116 Å and a α subcell could correspond to a 4L arrangement (Scheme 1) which is very rare (Karray et al., 2005). The other phases seemed to exhibit arrangements in 2L or 3L with β′ or pseudo β′ subcells which are more usually observed for triglycerides (Lopez et al., 2006). Thus, the covalent linkage of a monophosphate ddI did not seem to prevent this prodrug to organize like a triglyceride in anhydrous condition. The analysis of XRDT evolution was puzzling. Only, the use of coupling of DSC and XRDT (Ollivon et al., 2006) allowed to find a hypothesis that satisfied all observations. In the range $10 \leq T \leq 45$ °C, the coexistence of two phases with melting of a first species around 30 °C could not explain that (i) the intensity of the lines

Table 4
Size of ProddIP:DPPC 1:5 (mol:mol) liposomes before and after freeze-drying with different amounts of sucrose

	Before freeze-drying		After freeze-drying					
	Amount (%)	Size ± S.D. (nm)	Lipid:sucrose, 1:3 (w/w)		Lipid:sucrose, 1:5 (w/w)		Lipid:sucrose, 1:10 (w/w)	
			Amount (%)	Size ± S.D. (nm)	Amount (%)	Size ± S.D. (nm)	Amount (%)	Size ± S.D. (nm)
Population 1	37	156 ± 21	9	186 ± 80	36	323 ± 84	47	190 ± 29
Population 2	63	777 ± 90	91	1297 ± 858	64	1577 ± 321	53	936 ± 107



Scheme 1. Schematic representation of the anhydrous organization of ProddIP (/7) 4L (A) → 2L (B) transition between two different packings occurring during heating.

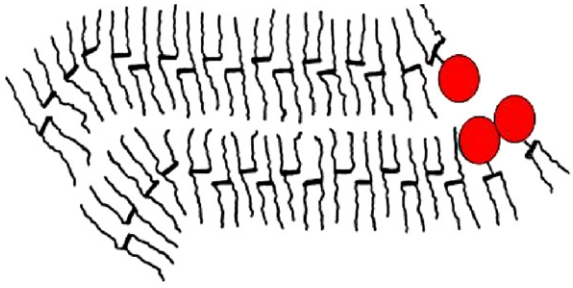


Scheme 2. Hypothesis concerning the influence of pH change on ProddIP solubility (s).

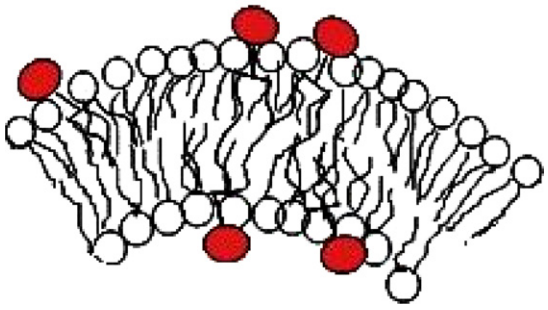
observed at small angles at about 58 Å stayed constant till about $T = 45\,^{\circ}C$, (ii) that an exotherm was observed at about 36 °C by DSC and (iii) the line observed at wide angles at about 4.15 Å disappeared around 30 °C. Then we concluded that a 4L → 2L transition between two different packings occurred during heating. At 10 °C the two species coexisted but the less stable one (4L) reorganized at the first DSC transition observed to transform into 2L. Only this species subsisted at high T up to T = about 45 °C.

To enhance the lymphatic absorption of ProddIP, lipidic formulations – i.e., emulsions, mixed micelles and liposomes – were explored. In a preformulation study, the solubilities of the prodrug in water at different pH, in sodium taurocholate micelles and in three different oils were investigated. As expected, thanks to the covalently linked glycerolipidic moiety, the solubility of the prodrug was very low in water. The influence of the pH on the water solubility of the prodrug could be explained by the change of the monophosphate group and the hypoxanthine group ionized as a function of the pH. At pH 8, the prodrug displayed a negative charge onto the phosphate as confirmed by the zeta potential. At pH 4, the nucleoside (and perhaps the phosphate group) may be protonated and the protonated prodrug would be more soluble in water (Scheme 2). In order to increase the water solubility of the prodrug, sodium taurocholate (20 mM) was added to obtain a micellar solution (CMC = 6.5 mM (Andrieux et al., 2004)). The choice of this surfactant results from the fact that this bile salt, at this concentration, is very well tolerated by intestinal cells (Meaney and O'Driscoll, 2000; Werner et al., 1996). In these conditions, the solubility of the prodrug was dramatically increased (10×, up to 108 µg/mL) but not yet sufficient to allow the development of a mixed micelle formulation for oral administration.

The formulation of the prodrug into emulsions was also investigated. Three oils of different compositions (soybean (majority of C_{18} fatty acids with two insaturations), almond (majority of C_{18} fatty acids with one insaturation), and fish liver oils (majority of very long chain fatty acids)) were chosen to solubilise the prodrug. Some differences in solubility were observed (Table 1) but the influence of the length and insaturation of chain triglycerides could not be explained. Surprisingly, the solubility of the prodrug in these oils was not enough to develop an emulsion for the oral administration of the ProddIP. The chemical structure of the prodrug and its organization as determined by XRDT seemed, indeed, very near to those of triglycerides which allowed expecting a better solubility in triglyceride based oils. But, the monophosphate-ddI moiety probably confers to the molecule a more hydrophilic character which is unfavourable to its solubilization into these oils (Scheme 3). Finally, we came to the conclusion that the structure of this new prodrug of ddI ($\log P = 2$) – with two hydrophobic chains and one hydrophilic group – possessed some analogy with the phospholipids. Therefore, a formulation based on DPPC liposomes was investigated for the inclusion of the prodrug. Noteworthy, DPPC was chosen because this molecule is composed of two palmitic acid chains just like the prodrug (Fig. 1). It was found that the prodrug could be efficiently incorporated (83–93%) into DPPC liposomes as determined by HPLC dosages. Then, DSC experiments were



Scheme 3. According to the organization of triglycerides ( ) in liquid state (Larsson, 1972), ProddIP molecules with their hydrophilic group ( ) may be excluded.



Scheme 4. Hypothesis on the insertion of ProddIP ( ) into DPPC ( ) lamellae.

used to confirm this encapsulation of the prodrug into DPPC lamellae and to evaluate their affinity as a function of the prodrug:DPPC ratio. Insertion of ProddIP into DPPC lamellae was confirmed by DSC experiments allowing also to identify the optimal prodrug:DPPC ratio. Until a 1:3 prodrug:DPPC ratio, the prodrug appeared to be inserted in DPPC multilamellae as suggested by the decrease of the onset temperature and enthalpy of the DPPC main transition. Further increase of ProddIP amount induced enthalpy enhancement, probably due to the crystallization of the prodrug perhaps stabilized by DPPC molecules in coexistence with the DPPC bilayers rich in ProddIP. Preliminary experiments of X-ray diffraction and microscopy seemed to confirm this hypothesis (data not shown).

Therefore, the liposomal formulation with a ProddIP:DPPC 1:5 ratio was selected for oral administration purpose. In this view, it was observed that DPPC liposomes were not efficient in protecting ProddIP from acidic degradation but that they even increased the sensitivity of ddI towards acidity, suggesting that this molecule was better exposed to the medium at the surface of the phospholipid membrane (Scheme 4). Indeed, the ddI and the spacer coupled to the diglyceride probably increased the length of the molecule in comparison with DPPC molecules which may explain the presence of ddI molecules at the surface of the DPPC liposomes and their exposition to the acidic medium (Fig. 1). This hypothesis has been confirmed by measuring the zeta potential which was dramatically negative (-57 ± 7 mV), suggesting again that the monophosphate ddI was, indeed, exposed towards the aqueous medium (in acidic medium, the prodrug loosed its hypoxanthine as didanosine did, data not shown). On the other hand, since ddI has very low solubility at acidic pH (Anderson et al., 1988), it was less sensitive to degradation at this pH, as compared with ProddIP liposomes.

Therefore, since the use of gastroresistant capsules have been considered, we have investigated the possibility to freeze-dry the suspension of ProddIP-loaded DPPC liposomes. This procedure has been successfully achieved by using sucrose as a cryoprotectant (at the optimal ratio lipid:sucrose of 1:10 w/w). In those conditions, it was found that the organization and size of the liposomes were kept intact even 15 days after their rehydration with water, thanks to their strong negative zeta potential.

In conclusion, this paper describes a new medicine of ddI which is based on the incorporation of a diglyceridic prodrug of ddI into DPPC liposomes. Further experiments are on the way to evaluate *in vitro* the absorption of this liposomal formulation of the didanosine glycerolipidic prodrug by enterocytes.

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