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A new nanomedicine based on didanosine glycerolipidic prodrug enhances the long term accumulation of drug in a HIV sanctuary

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ABSTRACT

New nanomedicines could improve drug accumulation in HIV sanctuaries and ameliorate their antiretroviral efficiency. In this view, we propose herein a combined strategy based on a biomimetic prodrug of ddI and its formulation in well-characterized lipid nanoobjects. The glycerolipidic prodrug of ddI (ProddINP) has been synthesized and its bulk structure was characterized. An appropriate formulation of this prodrug has been designed using a rational approach combining different physicochemical techniques. The high incorporation ratio of the prodrug into dipalmitoylphosphatidylcholine (DPPC) bilayers was determined by DSC. Then two liposome preparation methods were compared, with respect to size, incorporation yield and molecular/supramolecular organization of vesicles. The best liposomal formulation of ProddINP has been checked to keep intact the anti-HIV activity of ddI. This formulation was finally compared to ddI after oral route in rat. The animal experiments evidenced the increase of ddI blood half life (3-fold) and its enhanced accumulation as prodrug form at 24 h in numerous organs and especially intestine after administration of ProddINP in comparison with free drug. Finally, the tested liposomal formulation of ProddINP seems to be a promising approach to eradicate HIV infection from intestinal sanctuaries where the virus can concentrate.

1. Introduction

Major obstacle to HIV eradication in organism is viral sanctuaries, e.g. lymph nodes, CNS, and the intestine, which are only poorly accessible to anti-HIV compounds. It is the case of didanosine (ddI), a nucleoside reverse transcriptase inhibitor (NRTI). In this context, our approach was to synthesize a glycerolipidic prodrug of didanosine (ProddINP). It is expected that, due to the lipophilic character of this prodrug, better diffusion into these viral sanctuaries could occur.

The design of glycerolipidic prodrugs is an approach which was previously proposed to modify the biodistribution and increase the blood half life after oral administration of certain compounds by mimicking long chain triglycerides (Yatvin et al., 1999). This biomimetic strategy is, indeed, based on the metabolisation of long chain triglycerides in the gastrointestinal tract by the pancreatic lipase and co-lipase into a 2-monoglyceride and 2 fatty acids which are absorbed by enterocytes. After reesterification into triglycerides and packing into chylomicrons, these metabolites are secreted into the mesenteric lymph. The ability of this didanosine prodrug to be metabolised by pancreatine in vitro has been previously published evidencing the biomimetism of this approach (Lalanne et al., 2009).

Thus, in this study, we have designed a new formulation for encapsulating the glycerolipidic prodrug of didanosine. This prodrug was synthesized by covalent coupling to a 1,3-dipalmitoyl lipid through a succinate linker bound to the 5′-OH group of sugar

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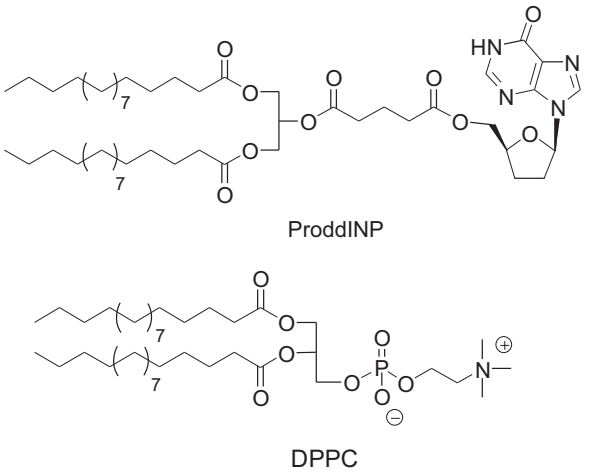


Fig. 1. ProddINP and DPPC chemical structures.

moiety (Lalanne et al., 2007) as depicted in Fig. 1. By adding a hydrophilic group as the didanosine in position 2 of glycerol, the prodrug is less hydrophobic than a triglyceride, suggesting a specific organization. Then the characterization of ProddINP has been performed using various physicochemical techniques. ProddINP polymorphism and its solubility in water, micellar solution and oils, were first determined. Since ProddINP presents a structural analogy with DPPC (Fig. 1), the solubility of ProddINP into hydrated DPPC lamellae was investigated by DSC. Then, DPPC liposomal formulations of ProddINP were prepared by the classical Bangham method (Bangham et al., 1974) and by an alternative solvent emulsion–evaporation technique (Sjostrom and Bergenstahl, 1992) with modification. Both preparation methods were compared, with respect to size, incorporation efficiency and supramolecular organization, using quasielastic laser light scattering (QELS), high performance liquid chromatography coupled with UV detection (HPLC–UV), scanning electron microscopy (SEM), cryo-transmission electron microscopy (Cryo-TEM), differential scanning calorimetry (DSC) and X-ray diffraction (XRD). These results allowed choosing the better formulation which was finally evaluated (i) in vitro for its anti-HIV-1 activity and (ii) in vivo by its pharmacokinetic and biodistribution after oral administration to rats.

2. Materials and methods

2.1. Products

All chemical reagents were obtained from Sigma–Aldrich (St Louis, USA) except ddI which was obtained from Bristol Myers Squibb (New York, USA). Solvents for the analytical section were obtained from Carlo Erba (Rodena, Italy). Almond oil and soybean oil were obtained from Cooper (Melun, France) and fish liver oil from SIRH (Fécamp, France). Sodium taurocholate, sodium chloride, dipalmitoylphosphatidylcholine (DPPC), and trehalose were obtained from Sigma (St Louis, USA). Sucrose, Na_2HPO_4 and NaH_2PO_4 were obtained from VWR Prolabo (Fontenay sous Bois, France). The ProddINP prodrug was synthesized as described in a previous study (Lalanne et al., 2007) by Artmolecules (Poitiers, France). Tritiated ddI was provided by Hartmann Scientific (Germany). Water was purified using a Synergy system from Millipore (France).

2.2. Polarized light microscopy

ProddINP was observed between crossed polarizers and with a λ/4 retarder in white light using a Nikon Eclipse E600 direct microscope (Champigny/Marne, France) equipped with a long focus objective (LWD 20 × 0.4; 1.2 mm) and a Nikon Coolpix 950 camera with a 1600 × 1200 pixels resolution.

2.3. High performance liquid chromatography coupled with UV detection (HPLC–UV)

The HPLC–UV set-up was similar to previously described (Lalanne et al., 2007). The limit of ProddINP detection (LOD) was 2.6 mg L^{-1} and limit of ProddINP quantification (LOQ) 7.8 mg L^{-1} (Lalanne et al., 2007). For prodrug solubility studies in oil, water and micellar solution of sodium taurocholate (20 mM), the prodrug (1 mg) was mixed with the solvent (200 μL of oils, water or micellar solution). The mixture was then ultrasonicated for 15 min and kept under mechanical stirring at 4 °C during 48 h before centrifugation (15 min, room temperature, 15,000 × *g*) (Bioblock Scientific 112® Centrifuge, Sigma, Steinheim, Germany). The pellet was then dissolved into ethyl acetate (1 mL) and diluted 10 times in methanol. The supernatant was diluted 10 times in ethyl acetate. The injection volume was 10 μL.

2.4. Preparation and incorporation yield of ProddINP-loaded DPPC liposomes obtained by Bangham's method (M1 vesicles)

Multilamellar vesicles (MLV) of dipalmitoylphosphatidylcholine (DPPC) incorporating ProddINP were prepared according to Bangham's method (Bangham et al., 1974) with modification and are called M1 vesicles hereafter. Briefly, the ddI prodrug (0.4 mg) and DPPC (3.25 mg) were dissolved into chloroform (0.4 mL). After mixing, the solvent was evaporated under reduced pressure using a rotavapor (Büchi Rotavapor R215, Büchi Vacuum Controller (*P* = 715 mPa until 0), Büchi Heating Bath B 491 (*T* = 50 °C), Büchi®, Rungis, France). Residual traces of organic solvent were evaporated under low vacuum, overnight in a freeze-dryer. The lipidic film was then rehydrated with heated phosphate buffer at 80 °C ($[HPO_4^{2-}]$ = 48 mM; $[H_2PO_4^-]$ = 28 mM; $[Na^+]$ = 144 mM; $[Cl^-]$ = 20 mM; pH 7.4) to obtain a final lipidic concentration of 2 mg mL^{-1} (prodrug + DPPC) for a 1:10 mol:mol ProddINP:DPPC ratio. The liposomal suspension was then filtered three times on 0.8 μm filter (Sartorius®, Palaiseau, France), at 80 °C, then cooled on ice. The same procedure was applied to prepare DPPC MLV. The suspension was directly injected in the HPLC–UV system to determine incorporation yield.

2.5. Preparation and incorporation yield of ProddINP:DPPC liposomes obtained by a solvent emulsion–evaporation technique (M2 vesicles)

ProddINP:DPPC liposomes, called M2 vesicles hereafter, were prepared by a solvent emulsion–evaporation technique as described before (Sjostrom and Bergenstahl, 1992; Gomez-Gaete et al., 2007) with modification. Briefly, DPPC (3.25 mg) was dissolved with ProddINP (0.4 mg) in chloroform (0.4 mL) before adding water (4 mL). The mixture was then ultrasonicated on ice twice for 1 min at 450 W using a Vibra cell sonicator (Bioblock Scientific, France) leading to the formation of an emulsion. The organic phase was then evaporated under reduced pressure using a rotavapor. The same procedure was applied to prepare unloaded DPPC liposomes. The incorporation yield was evaluated by HPLC–UV by direct injection of the suspension.

For animal experiments, M2 liposomes have been freshly prepared according to the previous method with some modifications. Briefly, DPPC (352.73 mg) was dissolved with ProddINP (38.65 mg) in chloroform (3 mL) before adding water (60 mL). The mixture was then ultrasonicated on ice bath for 35 min at 450 W (amplitude 70%). After solvent evaporation, the mixture was centrifuged

at 40,000 rpm during 1 h at 4 °C. The supernatant was discarded and the pellet was resuspended in 4 mL of previously filtered phosphate buffer at pH 8. This buffer was chosen to prevent acidic degradation of ddI and its prodrug in stomach of animals.

2.6. Size and zeta potential measurements

Mean diameters of liposomes were determined by quasielastic laser light scattering (QELS) with a N4 MD® Nanosizer (Coulter Electronic, Margency, France). Measurements were performed 15 min after liposomes preparation in order to ensure a steady state. Unimodal and Size Distribution Processor (SDP) results were recorded. When the population was multimodal, the mean diameter of each population and their standard deviation (SD) were given according to SDP results with the corresponding percent of light scattering intensity presented as amount (%) in Table 1.

Zeta potentials of M1 and M2 vesicles with a 1:10 (mol:mol) ProddINP:DPPC ratio, and DPPC vesicles were measured using a Zetasizer Nano Series® (Malvern Instruments, Malvern, United Kingdom). Suspensions were diluted 10 times in KCl solution (1 mM).

2.7. Freeze drying procedures

Sucrose was added to M1 vesicles as a cryoprotectant up to 2% (w/v). M2 vesicles were prepared using a trehalose/sucrose/water mixture (4 mL, 25% (w/v), for each of the cryoprotectant). Then, suspensions were quickly frozen in liquid nitrogen and freeze-dried. After reconstitution with satisfactory amount of water, the size and entrapment yield were determined as described above.

2.8. Scanning electron microscopy (SEM)

M1 and M2 vesicles composed of DPPC or 1:10 (mol:mol) ProddINP:DPPC prepared as described above were observed with a ZEISS SUPRA 55 VP microscope (Zeiss, Oberkochen, Germany) working not only at high vacuum but also at a controlled pressure inside the chamber (up to 133 Pa). A drop of suspension was deposited on a special conductive carbon tape and let to evaporate for a few minutes before being introduced in the SEM chamber. The gas pressure in the chamber was varied to optimize image quality. Finally only the high vacuum conditions were employed to take full advantage of the particularly good resolution of the SEM column at very low voltage. The accelerating voltage was set at 1 kV and the working distance close to 2 mm. Most images were acquired using the in-lens electron detector.

2.9. Differential scanning calorimetry (DSC)

Thermal analyses were conducted by DSC (DSC7, Perkin Elmer, Courtaboeuf, France). The prodrug melting behaviour was monitored in the 10–80 °C range at different scanning rates: 1 and 10 °C min^{-1}. The crystallization behaviour was monitored from 80 to 10 °C at the same rates. To highlight stable and metastable forms of the prodrug, a thermal treatment was applied: a 15 min heating at 50 °C, followed by a quick cooling down to 10 °C before monitoring the melting behaviour from 10 to 80 °C at 10 °C min^{-1}. Mixtures of ProddINP and DPPC at various ratios (0:1, 1:15, 1:10, 1:5; 1:3, 1:2, 1:0 (mol:mol)) were prepared by mixing adequate quantities of ProddINP and DPPC in 0.4 mL of chloroform. Organic solvent was then evaporated under reduced pressure using a rotavapor, and residual traces of organic solvent were evaporated under low vacuum. The lipidic film was then rehydrated with water with a 10:90 (w:w) lipid:water ratio before analysis. M1, M2 vesicles and freeze dried M2 vesicles composed of 1:10 (mol:mol) ProddINP:DPPC were prepared as described above before being ultracentrifuged at 40,000 × *g*, 30 min at room temperature. Each pellet was then loaded into a 40 μL pan before being analysed in the same conditions than the above mentioned lipidic films.

2.10. X-ray diffraction as a function of temperature (XRDT)

Small-angle (SAXS) and wide-angle (WAXS) X-ray scattering patterns of ProddINP during its heating at 1 °C min^{-1} were recorded simultaneously using two position-sensitive linear gas detectors and a microcalorimeter cell, MICROCALIX, used as a sample holder as previously described (Brubach et al., 2007). These experiments were performed with a fine-focus Cu anode source; Cu Kα (λ = 1.54 Å) radiation was selected and line focused by a multilayer mirror and collimated by slits. Detectors were calibrated with the crystalline β form of high-purity tristearin and with silver behenate, as previously described (Brubach et al., 2007). The scattered intensity was reported as a function of the scattering vector *q*:

$$q = \frac{4\pi \sin\theta}{\lambda} \quad (1)$$

where θ is half the scattering angle and λ the wavelength.

2.11. X-ray diffraction (XRD)

M1 and M2 vesicles composed of DPPC or 1:10 (mol:mol) ProddINP:DPPC were prepared as described above before being ultracentrifuged at 40,000 × *g*, 30 min at room temperature. The pellet was then loaded in calibrated Lindemann glass capillaries (diameter 1.5 mm, Glas-Muller, Berlin) prior to XRD analysis. SAXS and WAXS measurements on these samples were carried out at 20 °C using a monochromatic (8 keV) focused X-ray beam on the Austrian SAXS beamline at ELETTRA, Trieste, Italy. Simultaneous SAXS and WAXS scattering intensities were recorded using two one dimensional position-sensitive detectors (1024 channels, filled with argon–ethane mixture). X-ray data were collected by a National Instrument labVIEW supported data acquisition system.

Same detector calibration was applied. The scattered intensity was reported as a function of the scattering vector according to the same Eq. (1).

2.12. Cryo-transmission electron microscopy (Cryo-TEM)

A drop (3 μL) of M1 or M2 vesicles suspension (2 mg mL^{-1}) composed of 1:10 (mol:mol) ProddINP:DPPC was deposited onto glow-discharged quantifoil grids (Quantifoil Micro Tools, Jena, Germany) and vitrified in liquid ethane using a cryofixation device under humidity controlled condition. Cryospecimens were transferred to a Gatan 626 cryoholder (Gatan Inc., Warrendale, PA, USA) and examined at −180 °C with a JEM 2011 cryoTEM (JEOL, Tokyo, Japan) using a 200 kV acceleration voltage and a 30,000 or 50,000× magnification. Images were recorded with 1.6–4 μm of underfocus under low-electron-dose conditions on Kodak SO163 micrographs. Images (around 50) were analysed to determine the percentage of vesicles with different morphologies.

2.13. In vitro assessment of cytotoxicity and anti-HIV activity

Phytohemagglutinin-P (PHA-P)-activated peripheral blood mononuclear cells (PBMC) were treated by six concentrations of ProddINP in M2 vesicles reconstituted after freeze-drying (or unloaded liposomes as a control) at 100, 10, 1, 0.1, 0.01 and 0.001 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, viral stock titrated using PHA-P-activated PBMC, and 50% TCID50 calculated using Kärber's

Table 1
Sizes, zeta potential and entrapment yield (%) of liposomes: DPPC M1 vesicles (before and after filtration), 1:10 ProddINP:DPPC (mol:mol) M1 vesicles (before filtration, after filtration and after freeze drying), 1:10 ProddINP:DPPC (mol:mol) M2 vesicles (before and after freeze drying).

	Population	Amount [%]	Size [nm]	SD [nm]	Incorporation yield [%]	Zeta potential [mV]
DPPC	1	86	8915	1000	–	–
M1 liposomes (before filtration)	2	14	477	165		
DPPC	1	67	1365	237	–	1.7 ± 0.2
M1 liposomes (after filtration)	2	33	253	44		
ProddINP:DPPC	1	81	4210	607	99	–
1:10 (mol:mol)	2	19	290	40		
M1 liposomes (before filtration)						
ProddINP:DPPC	1	61	1864	338	12	−0.5 ± 0.5
1:10 (mol:mol)	2	39	379	114		
M1 liposomes (after filtration)						
ProddINP:DPPC	1	62	2168	319	12	–
1:10 (mol:mol)	2	38	318	183		
Freeze-dried M1 liposomes						
DPPC	1	100	250	43	–	–
M2 liposomes						
ProddINP:DPPC	1	100	187	75	99	−0.8 ± 5
1:10 (mol:mol)						
M2 liposomes						
ProddINP:DPPC	1	100	208	79	99	–
1:10 (mol:mol)						
Freeze-dried M2 liposomes						

formula (Kärber, 1931). Cell culture supernatants were collected at day 7 post-infection and stored at −20 °C. Viral replication was then measured by quantifying reverse transcriptase (RT) activity in these cell culture supernatants. In parallel, the cytotoxicity of the compounds was evaluated in uninfected PHA-P-activated PBMC using a 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; efficient doses (ED_{50}) and cytotoxic doses (CD_{50}) were calculated and represented the mean of both blood donors. Absence of unloaded liposomes cytotoxicity has been checked (data not shown).

2.14. Animal experiments

The animal experiments were carried out according to the principles of laboratory animal care and European legislation (recommendation 2007/526/EC) the protocol ethics were institutionally approved. Wistar rats weighting around 220 g were used for the studies and were purchased from Charles River laboratory (Domaine des Oncins, l'Arbresle, France). Rats had free access on water ad libitum and food but before treatment rats were fasted 16 h. For pharmacokinetic and biodistribution studies, rats were divided into two main groups one group was treated with ddI and the other group with ProddINP M2 liposomes. The control group contained rats that were not treated with any of the above drugs. The pharmacokinetic study was performed with catheterized rats ($n = 7$) on jugular veins. The biodistribution study was performed with rats ($n = 3$) without any chirurgical intervention. All of rats were sacrificed after anaesthesia with pentobarbital in i.p. route (50 mg/kg bw).

2.15. Pharmacokinetic studies

2.15.1. Sampling

ProddINP M2 liposomes or free ddI were administrated by oral route at equimolar doses of 45 mg of ddI/kg. Both of ddI and ProddINP liposomes were prepared in phosphate buffer at pH 8. Blood samples (400 μL) were collected from the catheter and transferred into citrated tubes at different times after initiation treatment (15 min, 30 min, 1 h, 2 h, 4 h, 8 h, and 24 h). Plasma samples were obtained by centrifugation (2000 × *g* for 10 min at room temperature) performed immediately after blood collection, and stored at −80 °C until quantifications of ddI. At 24 h the animals were sacrificed and their intestines were dissected for ddI content analysis.

2.15.2. High-performance liquid chromatography and tandem mass spectrometry

Practically, ddI plasmatic concentrations were determined as follows: briefly, after defrost, plasma samples obtained from treated animals were completed to 500 μL (validated dilution up to 1/10) with blank plasma and were centrifuged (18,000 × *g*, at room temperature for 10 min) and the internal standard (2-chloroadenosine) was added to supernatant. The extraction of ddI and its internal standard was performed by a solid phase technique using cartridges ATH according to several steps: conditioning (methanol, water), sampling, washing (water) and elution (methanol). The fraction of interest was then rescued and the solvent was evaporated under nitrogen flux. The residue was then resuspended in water/methanol (95/5). After centrifugation (9000 × *g* for 20 min), the supernatants were analysed by LC–MS/MS.

Moreover ddI concentrations in intestine were measured. Only two animals by group were considered for this analysis; they are chosen in the group according to their highest and lowest plasmatic ddI concentrations. Tissues were prepared as follows: after defrosted, the tissues were added with PBS (300 μL/100 mg of tissue) and homogenized with the tissue homogenizer Precellys 24 (Bertin Technologies, Montigny-le-Bretonneux, 78). After centrifugation (10,000 × *g*, 15 min, 5 °C), the supernatants were added with the internal standard. From that step, the preparation was the same as the plasmatic one (SPE extraction, evaporation, reconstitution).

Calibration and quality control samples were prepared and treated in the same way. Eighty microliters of the extracted samples were injected into the HPLC system Agilent HP100 (Agilent technologies). The separation was performed using a Zorbax SB C18 (150 × 2.1) column at a flow rate of 300 μL/min with a gradient elution (methanol/acetonitrile/water). Detection was performed with a mass spectrometer API 3000 (AB Sciex). DdI and chloroadenosine were detected in negative mode using the transitions, m/z 234.8 > 135 and m/z 284 > 168, respectively. The quantification process was performed using Analyst software 1.4 (AB Sciex, Les Ulis, France).

2.15.3. Organ distribution studies

Tritiated (^{3}H-) versions of ddI or ProddINP were used to study the organ distribution after oral administration in healthy rats. The radioactivity corresponded to 5 μCi/rat for ^{3}H-ddI and ^{3}H-ProddINP liposomes.

2.15.4. Radiolabelling of ProddINP

One mCi of tritiated ddI (30–45 Ci/mmol), labelled on C-2′ and C-3′ carbons was used for synthesis of radiolabelled ProddINP according to previous protocol (Lalanne et al., 2007). Tritiated ProddINP was obtained with a yield of 57% for a radioactivity of 0.08 mCi (5.25 mCi/mmol). The M2 liposomes were prepared according to the same method than for pharmacokinetic study using 0.91 mg of ^{3}H-ProddINP (solution at 33 μCi/mL in $CHCl_3$)

2.15.5. Sampling

ProddINP M2 liposomes or free ddI were administrated by oral route at equimolar doses of 45 mg of ddI/kg. Both of ddI and ProddINP liposomes were prepared in phosphate buffer at pH 8. After 24 h, rats of each group were sacrificed by cardiac puncture under general pentobarbital anaesthesia (50 mg/kg bw) and the blood samples were collected. The blood was centrifuged at 2000 × *g* for 10 min at room temperature, and the supernatant plasma was isolated. To 700 μL of plasma, 10 mL of Ultima Gold scintillant was added, and the mixture was vortexed vigorously for 1 min. The vials were kept aside for 1 h to allow the foam to dissipate and then counted in a beta counter (Beckmann, LS 6000TA; Beckman Coulter).

The organs, including the liver, lung, spleen, kidney, heart, muscle, intestine, testis, thymus, bone marrow and brain, were dissected. Fecal samples were processed in a similar manner: approximately 50–100 mg of the tissues was weighed and placed in the scintillation counting vials (in triplicate). Two milliliters of solvable was added into the vials containing tissue and kept in an incubator overnight at 50 °C to allow complete dissolution. Then 200 μL of hydrogen peroxide was added to each vial and kept in an incubator for 1 h at 50 °C to decolorize solutions. Later and after cooling solutions, 10 mL of Hionic-Fluor was added to the vials, which were vortexed vigorously for 1 min. The vials were kept aside for 1 h to allow the foam to dissipate. Separately, the urine samples (1 mL) were mixed with 10 mL of Ultima Gold, vortexed for 1 min, and set aside for 1 h for the foam to dissipate. The radioactivity present in the samples was measured (n = 3) using a beta counter. The results obtained for the biodistribution samples were converted and interpreted as percentage of administered dose per gram of tissue. Urine and feces were interpreted as total percentage dose The statistical comparisons were made using Student's test of an unequal variance using Origin 8 software.

3. Results

The objective of this study was first to encapsulate this ddI prodrug in a formulation which allows the administration of a 45 mg of ddI/kg dose by oral route in rat. This dose has been chosen according to the literature (Ray et al., 1990; Bramer et al., 1993; Manouilov et al., 1997). Considering the lipidic nature of the prodrug, lipid formulations (i.e. emulsions, lipid mixed micelles, solid lipid nanoparticles and liposomes) were tested and chosen formulations were then well-characterized.

3.1. Prodrug characterization

The didanosine prodrug was first extensively characterized concerning its molecular organization, thermal behaviour and solubility properties in different lipidic formulation media.

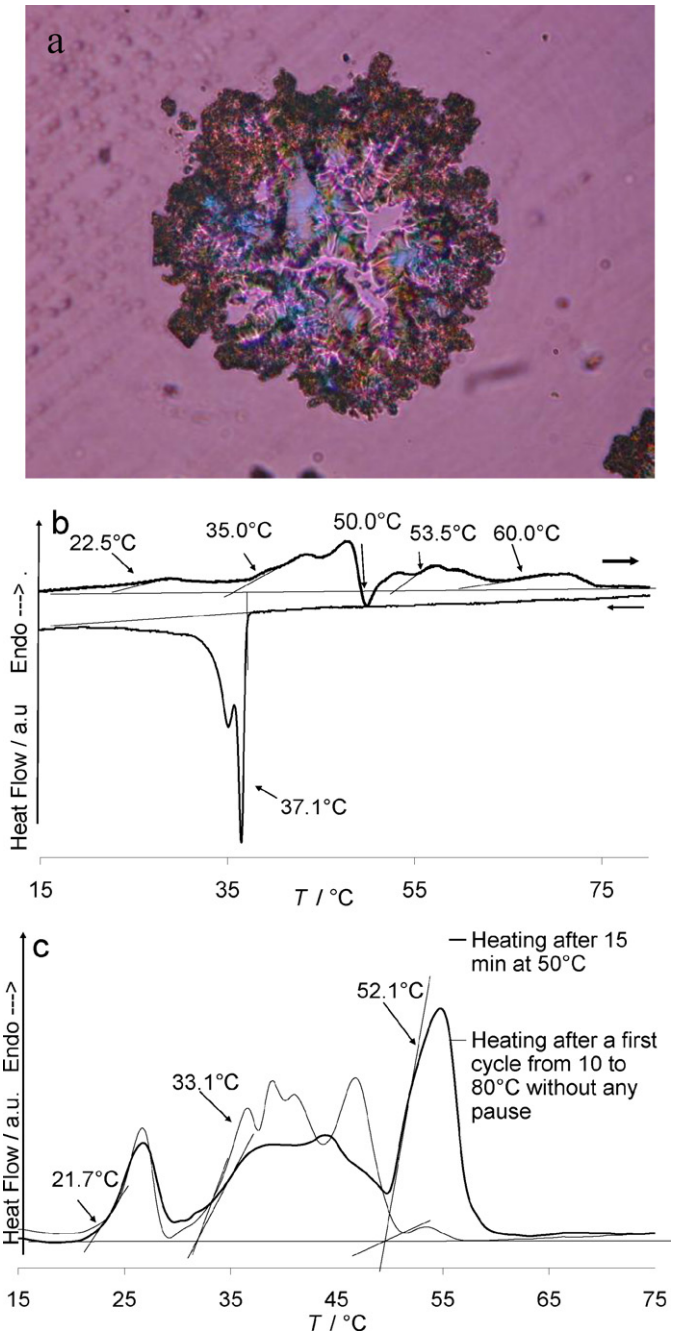


Fig. 2. Physico-chemical characteristics of ProddINP: (a) crystals of ProddINP in polarized light microscopy (scale bar represents 50 μm), (b) DSC recordings obtained by heating followed by cooling at 1 °C min^{-1} from 10 to 80 °C, and (c) DSC recordings of ProddINP, before and after thermal treatment (1/4 h at 50 °C) obtained at 10 °C/min from 10 to 80 °C.

3.1.1. ProddINP polymorphism

Polarized light microscopy observations showed crystallinity of ProddINP at room temperature (Fig. 2a). To determine its melting point and to explore its possible polymorphism, DSC experiments were performed at two heating rates: 1 °C (Fig. 2b) or 10 °C min^{-1} (Fig. 2c). Fig. 2b (top) shows a first endothermal event at 22.5 °C followed by a double endothermal event (beginning at 35.0 °C), an exothermal event at 50.0 °C and finally two endothermal events (at 53.5 °C and 60.0 °C). During the following cooling at 1 °C min^{-1} of the same sample, the DSC recording showed only two exothermal events beginning at 37.1 °C (Fig. 2b, bottom) which could correspond to the crystallization of the forms observed below 50 °C.

An immediate second heating of this sample showed changes in thermograms (data not shown) evidencing the influence of the temperature treatment. During the heating at 10 °C min^{-1} (Fig. 2c), the thermogram exhibited also a complex pattern with significant changes in number and relative intensities of thermal events in comparison with the lower heating rate, suggesting the coexistence of different crystalline forms of the prodrug. A thermal treatment composed of 3 steps: heating of the sample from 10 °C to 50 °C, isotherm during 15 min and rapid cooling down to 10 °C was applied to ProddINP (Fig. 2c). During heating after the thermal treatment, the enthalpy of the endotherm at 52.1 °C increased, contrary to those of other endotherms (observed at lower temperature), suggesting a modification of the prodrug organization (development of a more stable form at the expense of other crystalline forms of lower melting temperature) and evidencing the polymorphic behaviour as a function of temperature. X-ray diffraction diagrams (SAXS and WAXS) performed during the heating of the prodrug at 1 °C min^{-1} (data not shown) confirmed the prodrug reorganization at 50 °C and the complete melting of the new polymorphic forms above 80 °C, already observed by DSC. At 20 °C, SAXS analysis showed a wide peak centered on 75.2 Å.

3.1.2. ProddINP solubility

ProddINP solubility in different aqueous solutions or oils was determined by HPLC–UV. Independently of the pH, the prodrug solubility in water was found very low (<3 μg mL^{-1}), below the limit of detection (LOD). Solubility was slightly increased in a 20 mM sodium taurocholate solution (43 μg mL^{-1}). At this concentration this bile salt is very well tolerated by intestinal cells (O'Driscoll, 2002) and could be used to prepare micellar solution of drugs. The best solubility was found in soybean oil (237 μg mL^{-1}, i.e. 0.03% (w:w)) and in fish liver oil (244 μg mL^{-1}, i.e. 0.03% (w:w)). The difference between oil compositions (soybean (majority of C18 fatty acids with two insaturations), almond (majority of C18 fatty acids with one insaturation), and fish liver oils (majority of very long chain fatty acids)) seems to have no clear influence on prodrug solubility. These results suggested that sodium taurocholate mixed micelles and emulsions composed of these oils were not appropriate lipid formulations for loading of the prodrug in formulation for in vivo evaluation.

3.1.3. Incorporation of ProddINP into hydrated DPPC lamellae

ProddINP incorporation into hydrated DPPC lamellae was investigated by DSC. Transitions of DPPC mixed with increasing amounts of ProddINP were followed at 10 °C min^{-1} (ProddINP:DPPC (mol:mol) 0:1, 1:15, 1:10, 1:5, 1:3, 1:2, 1:0) (Fig. 3). The thermogram of hydrated ProddINP (1:0) prepared according to the same procedure as ProddINP:DPPC mixtures was also recorded (onset of the 3 main endotherms = 33.1, 37.7, 48.7 °C). An endotherm at 41.4 °C (ΔH = 60 J g^{-1}), corresponding to the melting transition of the acyl chains, was observed during the heating of pure DPPC as expected (Grabielle-Madelmont and Perron, 1983; Small, 1986). When the proportion of ProddINP increased in mixtures, a significant decrease of both the onset temperature and the enthalpy of DPPC chain melting transition was observed up to a 1:10 (mol:mol) ProddINP:DPPC ratio (onset = 39.7 °C, ΔH = 40.5 J g^{-1} of DPPC). Above this ratio, two main endotherms with increasing enthalpies were observed: one at a constant temperature of 39 °C and a second with increasing onset temperature (onset: 45.0, 47.3 and 52.1 °C for 1:5, 1:3 and 1:2 (mol:mol) ProddINP:DPPC ratios, respectively), tending towards the melting of ProddINP crystals. This suggested that ProddINP was not completely incorporated into DPPC bilayers at these ratios. Thus, the limit of prodrug solubility into hydrated DPPC lamellae seemed to occur at a ProddINP:DPPC ratio of 1:10 (mol:mol) (12.31% (w:w)) which was chosen for further preparation of liposomes.

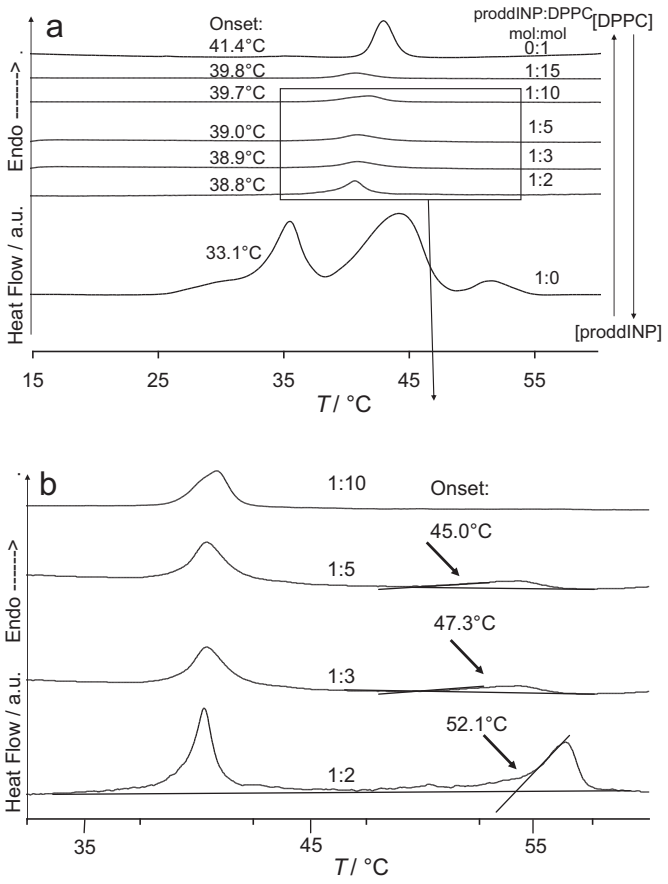


Fig. 3. Influence of ProddINP insertion on DPPC thermal properties: (a) global DSC records of DPPC thermogram with increasing amounts of ProddINP and (b) enlargement of some DSC records (ProddINP:DPPC (mol:mol) 1:10, 1:5, 1:3, 1:2).

3.2. Preparation of ProddINP liposomes

3.2.1. Preparation of ProddINP:DPPC liposomes by Bangham's method (M1 vesicles)

Multilamellar vesicles (MLV) of 1:10 (mol:mol) ProddINP:DPPC were first prepared by the classical Bangham method with a good incorporation yield (99%) confirming DSC results. In order to decrease and to homogenize MLV size, 3 filtrations were successively performed. Filtered MLV (M1 vesicles) were polydispersed with a population around 380 nm and another one of relatively large size (around 2 μm) (Table 1). Zeta potential was found to be close to neutrality, and the liposome suspension was not stable upon one day storage. Moreover, the incorporation yield was dramatically decreased (12% of the initial amount) after the filtration step. To make possible the storage of the liposomal suspension, we then investigated its freeze-drying using different sucrose concentrations. When using 2% (w/v) sucrose solution, the two size populations remained unchanged after rehydration (Table 1) as well as the amount of ProddINP incorporated.

3.2.2. Preparation of ProddINP:DPPC vesicles by solvent emulsion–evaporation method (M2 vesicles)

To overcome the loss, due to filtration, of ProddINP in liposomes prepared by Bangham's method, an alternative emulsion–evaporation technique was investigated to formulate ProddINP:DPPC at the optimal ratio. Thanks to this preparation method, no filtration was needed to obtain a single population of small size: around 200 nm according to QELS analysis (Table 1) with high incorporation yield (99%). As for M1 vesicles, the size of M2

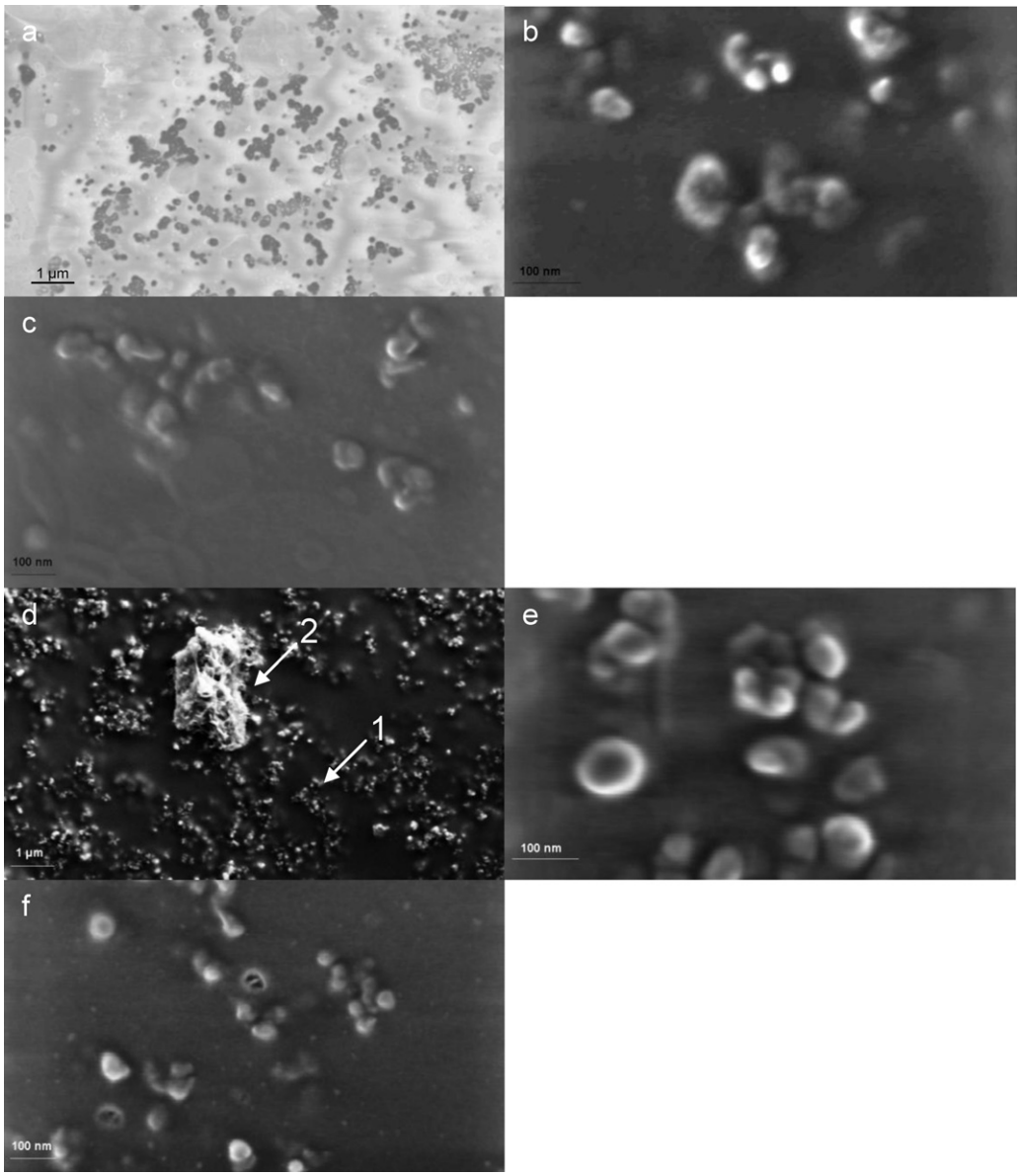


Fig. 4. SEM pictures of (a and b) DPPC M1 liposomes by Bangham method, (c) DPPC M2 vesicles, (d and e) ProddINP:DPPC (mol:mol) 1:10 M1 liposomes, (f) ProddINP:DPPC (mol:mol) 1:10 M2 vesicles. Scale bars represent 1 μm (a and d) and 100 nm (b, c, e, and f).

vesicles was not stable upon storage, as explained by their zeta potential close to neutrality. For long term storage, M2 vesicles were freeze-dried using a trehalose/sucrose solution (containing 25% (w/v) of each cryoprotectant). After rehydration the vesicles size was recovered as well as the amount of incorporated ProddINP (Table 1).

3.3. Molecular and supramolecular organization of ProddINP liposomal formulations

In this section, the influence of the prodrug incorporation and of the liposome preparation method on the molecular and supramolecular organization of ProddINP liposomes is investigated.

3.3.1. SEM

SEM observations of DPPC M1 vesicles revealed large aggregates (Fig. 4a) in coexistence with very small spherical objects of about 100 nm (Fig. 4b). For DPPC M2 vesicles (Fig. 4c), only spherical objects of about 100 nm were visualized. M1 vesicles of ProddINP:DPPC were shown as a coexistence of small spherical vesicles (around 100 nm, Fig. 4d, arrow 1, and e) and bigger aggregates (around 3 μm, Fig. 4d, arrow 2). The observation of M2 vesicles loaded with ProddINP showed only one population around 100 nm (Fig. 4f).

3.3.2. Cryo-TEM

Cryo-TEM observations of ProddINP:DPPC liposomes prepared by both methods (Fig. 5) showed similar small vesicles (67% of M2 and 82% of M1 vesicles were smaller than 150 nm) which are polydisperse in size and lamellae number and with some unexpected undulations in lamellae. Noteworthy, the experimental conditions used excluded vesicles or aggregates larger than 300 nm from the observations.

3.3.3. DSC

Fig. 6 shows the comparison of the DSC recordings corresponding to M1, M2, and rehydrated freeze-dried M2 vesicles of

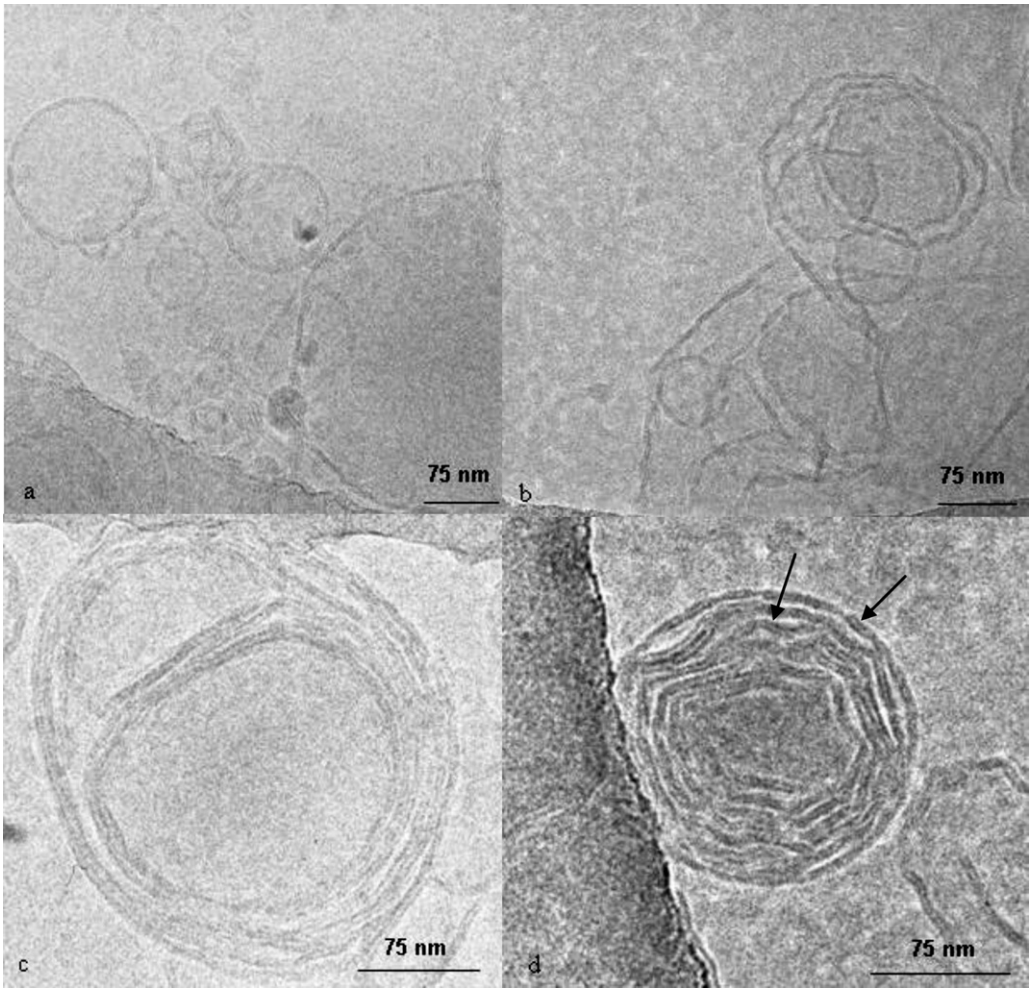


Fig. 5. Cryo-TEM pictures of 1:10 ProddINP:DPPC (mol:mol) M1 (a and c) and M2 (b and d) vesicles. (a) and (c) were taken with 1.6 μm, (b) with 2 μm and (d) with 4 μm of underfocus (for this image, the contrast was increased at the expense of resolution). Magnification used was 50,000× (a) or 30,000× (b). The arrows indicate the undulations of bilayers.

ProddINP:DPPC. No significant difference was observed on the onset temperature of each endotherm corresponding to mixed ProddINP:DPPC bilayers. Although the melting transition observed for freeze-dried M2 vesicles was a little wider than the 2 others, no peak corresponding to ProddINP melting was evidenced.

3.3.4. XRD

Finally, the influence of the preparation method on the organization of the vesicles was also examined by X-ray diffraction experiments performed at 20 °C (Fig. 7). SAXS and WAXS patterns of pure DPPC vesicles prepared using the Bangham method (Fig. 7a and b) presented the already reported diagrams (Forte et al., 1998; Carion-Taravella et al., 2002) at room temperature and in excess of water. DPPC was in the lamellar gel phase Lβ′ (*d*-spacing = 64 Å) in which extended chains were tilted with respect to the bilayer plane and arranged in a pseudohexagonal subcell. The X-ray diffraction diagrams of DPPC vesicles prepared by solvent emulsion–evaporation technique (Fig. 7c and d) showed roughly the same organization but with wider SAXS peaks suggesting some disorder in bilayer stacking. SAXS and WAXS recordings of ProddINP:DPPC mixtures prepared by both techniques (Fig. 7e and f: M1 vesicles and g and h: M2 vesicles) showed the same lamellar organization. The wider SAXS peak observed with M2 vesicles suggested less organization within the bilayer stacks obtained by this method and/or less multilamellar liposomes. Compared to pure DPPC, the increase of the interlamellar distance (64 Å → 71.1 Å), the change from the pseudohexagonal subcell to a hexagonal subcell (4.23 Å) and the wider WAXS peaks evidenced prodrug incorporation. Both SAXS and WAXS experiments were in agreement with a perfect insertion of ProddINP into DPPC bilayers.

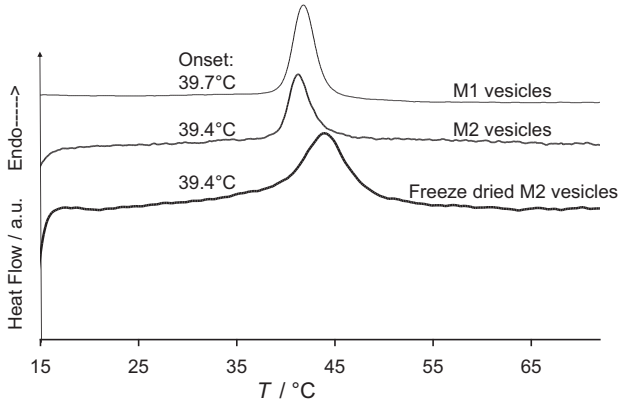


Fig. 6. Comparison of DSC recordings obtained by heating from 10 to 80 °C at 10 °C min^{-1} of 1:10 ProddINP:DPPC (mol:mol) M1, M2 and freeze-dried M2 vesicles.

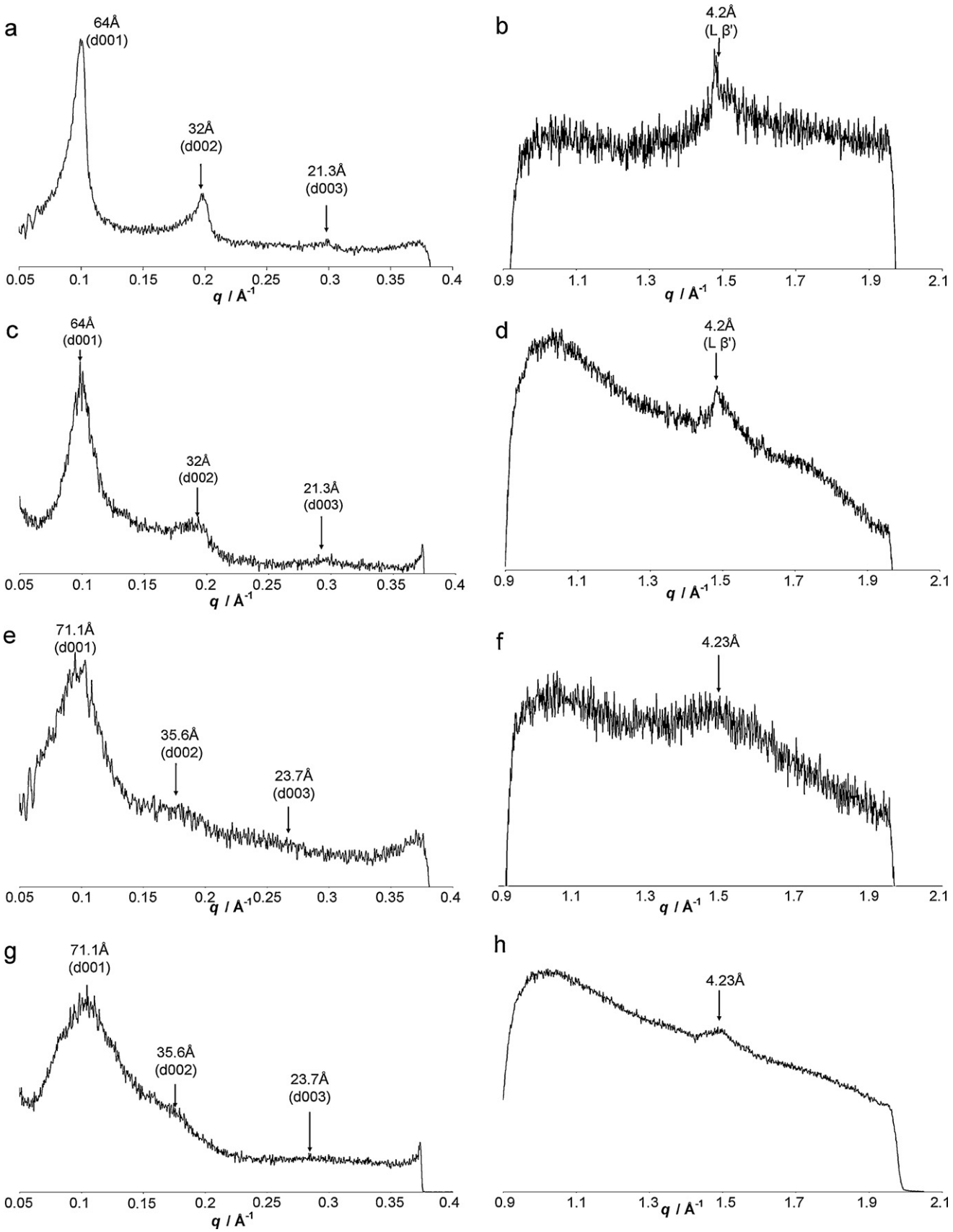


Fig. 7. SAXS (a, c, e, and g) and WAXS (b, d, f, and h) recordings of (a and b) DPPC M1 vesicles, (c and d) DPPC M2 vesicles, (e and f) 1:10 ProddINP:DPPC (mol: mol) M1 vesicles, (g and h) 1:10 ProddINP:DPPC (mol: mol) M2 vesicles.

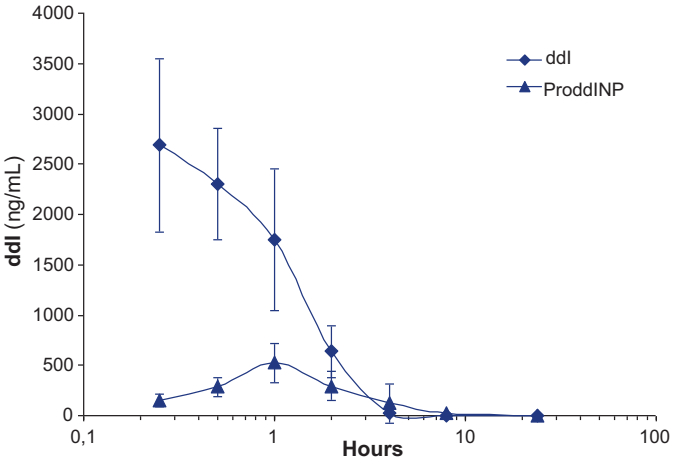


Fig. 8. Mean (±SD) plasma concentrations of ddI following oral administration of 45 mg/kg ddI (♦) and an equimolar dose of ProddINP (△).

The coexistence with free ProddINP lamellae (*d*-spacing 75.2 Å) was improbable and moreover not evidenced by SEM and DSC analyses.

3.4. In vitro cytotoxicity and antiviral activity of ProddINP in DPPC liposomes (M2 vesicles)

The anti-HIV activity and cytotoxicity of freeze-dried M2 vesicles were determined towards PHA-P-activated PBMC. M2 vesicles presented anti-HIV-1-LAI effects similar to those obtained with M1 liposomes (M2: ED_{50} = 9.9 μM, CD_{50} > 100 μM vs. M1: ED_{50} = 2.8 μM, CD_{50} > 12 μM, Lalanne et al., 2007) and with ddI alone (ED_{50} = 1.2 μM, CD_{50} = 40 μM, Lalanne et al., 2007). These results showed that the preparation method had no influence on the anti-HIV ddI efficacy. Noteworthy, these in vitro experiments were performed one month after freeze-drying of the M2 vesicles and the activity was entirely recovered, as compared to the antiviral efficacy of ddI free. These data are not trivial because, in general, the major limitation to the prodrug approach is the ability of the relevant enzymes to activate the prodrug, allowing the release of the active compound. In view of these results, two hypotheses may be proposed: (1) the prodrug could penetrate into the cell and release the ddI intracellularly which is then phosphorylated to display the antiviral activity or (2) the prodrug could release the ddI in the extracellular medium; the nucleoside analogue is then further phosphorylated after intracellular capture. The fact that the prodrug nanoformulation did not significantly modify the antiviral activity, neither decreasing nor increasing suggests that the second hypothesis is more probable.

3.5. Animal experiments

For animal experiments, fresh M2 vesicles were prepared with optimized conditions and exhibited mean diameter of 131 nm (polydispersity index = 0.1).

3.5.1. Pharmacokinetic study

M2 liposomes and ddI solution were administered by oral route to rats. Plasma ddI concentrations were determined as a function of time (Fig. 8). The plasma concentration–time curve of ddI, in rat treated with free ddI, was characterized by a decrease in two steps (Fig. 8, diamonds), as already described in mice plasma (Manouilov et al., 1997). The plasma drug time course in the group of ProddINP M2 liposomes-treated rats seemed to follow a typical logarithmic curve observed after oral administration (Fig. 8, triangles). The results showed a lower plasmatic exposure of ddI after administration of ProddINP (AUC_{0-Inf} = 3569 ng h mL^{-1}) comparatively to free ddI (AUC_{0-Inf} = 1520 ng h mL^{-1}), especially between the time points 15 min and 2 h. On the contrary, at the ultimate time points (i.e. 4 and 8 h), the ddI plasmatic concentrations were, respectively, 6 and 5-fold, higher for the prodrug than after the administration of ddI as a free molecule. ProddINP-loaded liposomes presented a higher terminal elimination $t1/2$ (2.37 h) and T_{max} (1 h) as compared with the free drug (0.76 and 0.25 h, respectively). Noteworthy, in this study, only ddI was measured in the plasma whereas the concentrations of the other ddI based molecules (i.e. ProddINP and metabolites) were not assessed. Because these molecules could release only part of ddI molecules, we hypothesized that the concentration of all ddI molecular species was probably higher than suggested by these results.

3.5.2. Organ distribution

Tissue distribution of radioactivity was determined in rats after single oral dosing of either ^{3}H-ddI or ^{3}H-ProddINP liposomes. The evaluation at 24 h after the administration of either the prodrug M2 liposomes or the ddI free showed in general higher tissue distribution of the radioactivity for the liposomal formulation and especially in the intestine, the spleen and the testicles; only in the brain and the thymus radioactivities were equivalent for both treatments (Fig. 9a). Moreover, radioactivity recovered in plasma was 4-fold higher after prodrug administration than after free ddI, thus confirming that the ddI concentration measured in the pharmacokinetic study (time point 24 h) represented only a small fraction of the total pool of ddI. The urinary excretion of radioactivity provided by M2 liposomes was increased compared to that of free ddI whereas the feces excretion was similar after both treatments (Fig. 9b). In order to know if the high level of radioactivity found in the intestine after M2 liposomes administration corresponded or not to ddI released from the prodrug, a dosage of ddI was performed in this tissue 24 h after oral administration. These dosages showed a lower ddI concentration (molar ratio = 28%) in intestine after prodrug administration in comparison with the concentration obtained with free ddI. This suggested that the majority of the ddI localized in intestine after ProddINP M2 liposomes administration did not correspond to ddI free but rather to a prodrug form (perhaps different of ProddINP).

4. Discussion

With the aim to improve HIV treatment, we have designed a new nanomedicine loaded on a glycerolipidic prodrug of didanosine in order to increase its half life and its biodistribution, especially in HIV sanctuaries.

First the prodrug and its formulation have been extensively characterized. Since the polymorphism of the triglycerides is well known, we have investigated the polymorphism of ProddINP. Thanks to DSC, XRDT and microscopy experiments, it was observed that ProddINP was crystalline and existed in various forms: at least 3 metastable forms and 3 more stable forms. Temperature is, therefore, a key parameter for the storage of the prodrug and its formulation. In order to avoid the influence of the prodrug polymorphism on its formulation, we employed two liposomes preparation procedures initially based on the solubilization of ProddINP into chloroform and mixing with DPPC.

The ability of ProddINP to be solubilized into triglycerides composing oils was determined by HPLC–UV. However, despite the diglyceridic nature of the prodrug, solubilities of ProddINP in soybean oil and in fish liver oil were very low (0.03% (w:w)). The poor solubility of ProddINP in various oils could be attributed to the amphiphilic character of ProddINP which is constituted of a dipalmitoylglyceride linked to the more hydrophilic moi-

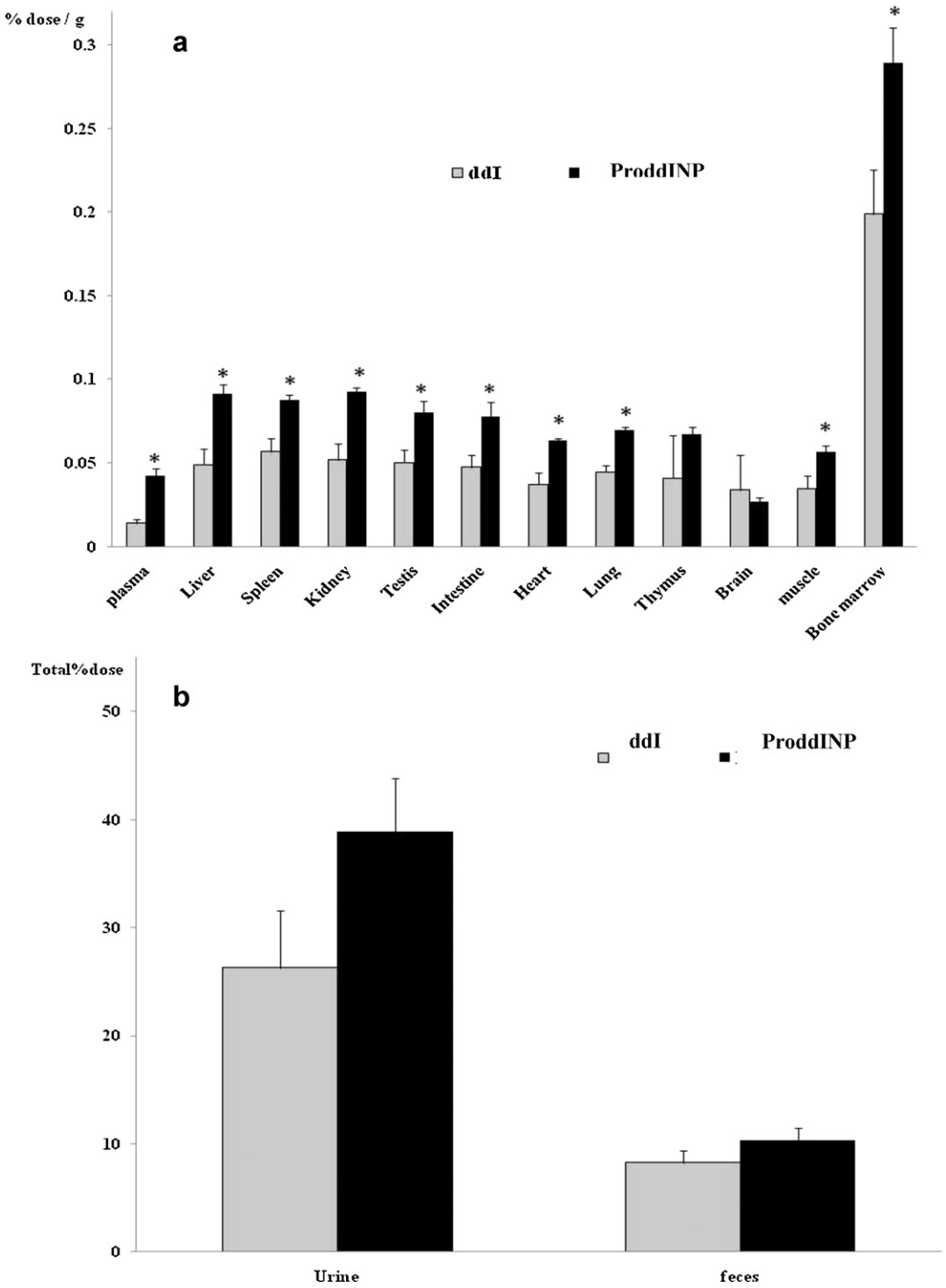


Fig. 9. (a) Plasma and organ distribution of radioactivity after the oral administration of 45 mg/kg ^{3}H-ddI (grey) and an equimolar dose of ^{3}H-ProddINP (black). (b) Urinary excretion and fecal samples radioactivities after the oral administration ($^{*}p < 0.05$).

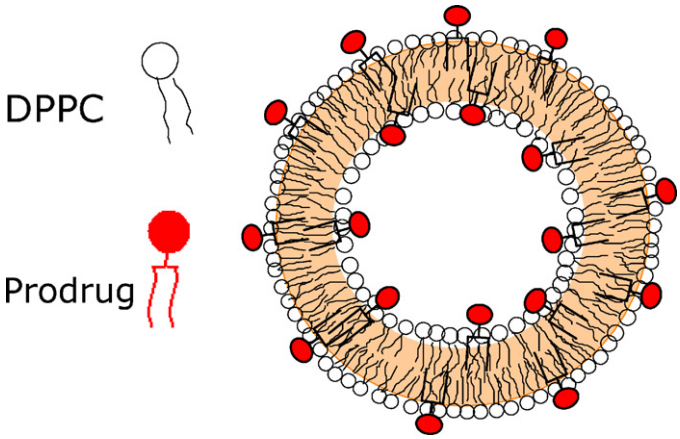
ety didanosine, known to be polar. It has to be noted that the octanol/water partition (Log *P*) could not be determined accurately because of the very low solubility of ProddINP in water too. Besides, a preliminary assay was performed in order to solubilize ProddINP using sodium taurocholate which form mixed micelles with glycerides in the gastroduodenal tract during triglyceride digestion. However, the solubility of ProddINP in this formulation was found to be very low too.

The structural analogy of the prodrug with phospholipids and especially DPPC which possess the same hydrocarbon chains (Fig. 1) opened the possibility to formulate the prodrug into liposomes. To ensure vesicle formation, drug incorporation must, however, be at a concentration below a critical ratio. Above this ratio, the mixed (phospholipid/drug) bilayer can be disrupted or saturated, leading to the formation of another phase composed of drug in excess (in mixture with phospholipids or not). Therefore, the incorporation of ProddINP into DPPC lamellar phase was studied by DSC (Fig. 3). As expected, a good incorporation (ProddINP:DPPC (mol:mol) 1:10, 12.3% (w:w)) was found. The insertion of ProddINP into DPPC bilayers was confirmed by SAXS and WAXS experiments (Fig. 7).

Because of this encouraging result to achieve a dosage form with requested ddI dose, DPPC liposomal formulations were chosen for ProddINP incorporation, first using the classical Bangham method (M1 vesicles). Before filtration, this methodology provided MLV liposomes with a large size and high polydispersity as observed by QELS, SEM and Cryo-TEM. The major population of loaded DPPC liposomes exhibited 4210 ± 607 nm as hydrodynamic mean diameter. Carey et al. (1983) reported that the dispersed phase of a typical duodenal o/w emulsion was largely composed of stable vesicles around 0.5 μm in size. Moreover, pancreatic lipase only attains its full catalytic activity in the presence of these o/w emulsions, since the enzyme is "activated" by interfacial binding (Ferreira and Patton, 1990). In order to decrease the size of M1 liposomes (chosen size ≤ 0.5 μm) prodrug-loaded liposomes were filtrated. But after filtration, the size of ProddINP-loaded liposomes remained too large (1864 ± 338 nm) (Table 1; Fig. 4d and e) and most of the prodrug was lost upon filtration (from 99% incorporation yield before filtration to 12% after filtration). This was attributed to the probable adsorption of ProddINP onto the filters, following disruption of the bilayers due to the extrusion of the vesicles and leading to the release of the prodrug. This is the reason why we have considered an alternative methodology for preparing ProddINP-loaded liposomes with high loading yield, i.e. without filtration. Although extrusion (Olson et al., 1979) and high pressure homogenization (Mayhew et al., 1984) are widely used techniques, they both require filtration or extrusion. Sonication (Saunders et al., 1962) (6 cycles of 2 min at 500 W), as described by Huang (1969) and Lesieur et al. (1993) is, on the contrary, the only method allowing to obtain small SUV liposomes (50 nm) without filtration. However, with this method, the incorporation yield is usually very low (1%, Szoka and Papahadjopoulos, 1980). Sonication was also described to prepare SLN (solid lipid nanoparticles) (Sjostrom and Bergenstahl, 1992) and polymeric nanoparticles (Gomez-Gaete et al., 2007). In the present study, a combination of emulsion/evaporation technique with sonication has been successfully applied for incorporation of ProddINP into DPPC liposomes (M2 liposomes) with appropriate size as observed by QELS and SEM (Table 1; Fig. 4f) and with a very high incorporation yield (99%).

Structure and morphology of M1 and M2 liposomes were then investigated by SEM, Cryo-TEM, DSC and XRD in order also to study their supramolecular organization in the presence of ProddINP. Cryo-TEM observations (Fig. 5) evidenced that both M1 and M2 preparation procedures allowed the formation of similar uni- and oligo-lamellar vesicles when ProddINP was added. The presence of a large percentage (95%) of unilamellar vesicles was unexpected, especially with the M1 Bangham preparation method. Interestingly, some undulations in the mixed lamellae were observed confirming the incorporation of ProddINP molecules into the DPPC bilayer of the liposomes prepared by both methods. These undulations were possibly the indication of some disorganization of the lamellar phase facilitating the formation of unilamellar vesicles. X-ray diffraction data (Fig. 7) have verified that the insertion of ProddINP caused, indeed, a partial disorganization of DPPC lamellar phase as evidenced by wider SAXS peaks. No significant difference between the interlamellar or interchains distances determined by XRD (Fig. 7) or the melting temperatures measured by DSC (Fig. 6) of ProddINP:DPPC liposomes as prepared by both methods could be observed, thus suggesting similar supramolecular organization. This observation evidenced that the conditions of preparation (i.e. heating during M1-type liposomes preparation) had no influence on the resulting molecular organization. In a nutshell, all these data allowed concluding that all didanosine prodrug molecules were inserted within DPPC lamellae and that M1 and M2 objects could be described as uni- or oligo-lamellar mixed liposomes.

Although the supramolecular organization of M1 and M2 liposomes appeared to be similar, their mean diameter and size distribution were found very different as measured by QELS and SEM: with ProddINP:DPPC M1 liposomes (Fig. 4d and e; Table 1), a population of small liposomes around 100–200 nm was in coexistence with big aggregates whereas ProddINP:DPPC M2 liposomes (Fig. 4f; Table 1) displayed only a single population of small and spherical objects. Moreover, freeze-drying of M1 and M2 liposomes could not be obtained in the same conditions. Trehalose was added as an additional cryoprotectant for M2 vesicles. This adjuvant, known to be a powerful cryoprotectant (Crowe et al., 1985; Harrigan et al., 1990), was needed when very small liposomes with high specific surface area were freeze-dried to avoid their aggregation. The difference in liposome size as observed with M1 and M2 liposomes were consistent with X-ray diffraction results showing enlarged diffraction peaks in M2 liposomes samples.



Scheme 1. Schematic representation of ddI prodrug localization into phospholipidic bilayers of liposomes.

Additionally, the difference between M1 and M2 ProddINP liposomes may be explained by the ratio of ProddINP incorporation in both formulations (12% in M1 vs. 99% in M2 liposomes). It is hypothesized that the amphiphilic character of the prodrug which would be inserted into phospholipids with ddI (polar group of the prodrug) exposed towards the aqueous medium (Scheme 1) could prevent the vesicle aggregation.

These results combined with those of antiviral activity suggested that the best formulation of ddI prodrug was M2 liposomes. After optimization of the preparation method, a liposomal suspension with small size (comparable to typical duodenal vesicles) and adequate drug loading was available for animal experiments. The pharmacokinetic experiments evidenced the increase of T_{max} (4-fold) and blood half life (3-fold) of ddI after the oral administration of the prodrug in comparison to the free drug (Fig. 8). A previous study (Manouilov et al., 1997) has evidenced a decreased ddI concentration in plasma due to an increased ddI concentration in lymph when ddI was administered as a lipid prodrug. Thus, we can hypothesize a similar behaviour with our ProddINP formulation.

Moreover, 24 h after the prodrug administration (Fig. 9), the localization of the radioactivity associated with ddI was clearly enhanced in numerous organs and especially intestine and spleen which are important sanctuaries of HIV and also in testicles known as a possible viral reservoir (das Neves et al., 2010). However, only a low part of the radioactivity found in intestine corresponded to free ddI. These results could be explained by a possible retention of the lipid prodrug in the cell membranes in general and especially at the level of the intestinal epithelium, followed only later on a progressive released in the lymph and then the blood. In this study, we have already optimized the type of lipidic formulation used, the ratio of prodrug/phospholipid employed as well as the liposome preparation method. However, according to previous studies (Wang et al.,

2002; Shaaya et al., 2003), the modification of the length of the acyl chains of the prodrug is an option susceptible to modify and perhaps to improve the pharmacokinetic parameters. Of course, this approach needs to perform the synthesis of new prodrugs and to investigate their formulation before testing pharmacokinetic and biodistribution.

In conclusion, we have designed a new nanomedicine based on a biomimetic approach thanks to a glycerolipidic prodrug. After extensive characterization of the prodrug and of its liposomal formulations, the best formulation has been selected for in vitro and in vivo evaluation. This new nanomedicine allowed to preserve ddI anti-HIV activity and to enhance blood half-life and intestine accumulation. All these results suggested that the tested liposomal formulation of ProddINP could be a promising approach to eradicate HIV infection from intestinal sanctuaries where the virus can concentrate. Further in vivo experiments will investigate the antiretroviral efficacy of this formulation, focusing in particular on HIV sanctuaries like intestine and lymph nodes.

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