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Research paper

Development and characterization of a novel lipid nanocapsule formulation of Sn38 for oral administration

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ABSTRACT

The purpose of this work was to encapsulate 7-Ethyl-10-hydroxy-camptothecin (Sn38) in lipid nanocapsules (LNCs) using phase inversion-based method in order to deliver Sn38 by oral route. LNCs were prepared by a low-energy emulsification method and were characterized for size, polydispersity index (PDI), surface charge, drug payload, *in vitro* drug release, and storage stability. Moreover, in view of an oral administration, *in vitro* stability in gastrointestinal fluid and permeability across Caco-2 cells were tested. Sn38-loaded LNCs with a mean particle size of 38 ± 2 nm were obtained. The particles displayed a narrow size distribution and a drug payload of 0.40 ± 0.07 mg/g of LNC dispersion. *In vitro* stability in simulated gastric and intestinal media was also observed. Finally, Sn38-loaded LNCs improved permeability of Sn38 across Caco-2 cells ($5.69 \pm 0.87 \times 10^6$ cm s^{-1} at 6 h vs $0.31 \pm 0.02 \times 10^6$ cm s^{-1}) and intracellular concentration compared with free Sn38. In conclusion, Sn38 nanocarriers have been developed and display a strong potential for oral administration.

1. Introduction

7-Ethyl-10-hydroxy-camptothecin (Sn38), a biologically active anticancer drug, is metabolized from irinotecan hydrochloride (CPT-11) by carboxylesterases in the liver and in tumors. Sn38 inhibits the release of single-strand DNA breaks mediated by topoisomerase I by stabilizing cleavable complex [1–3]. This metabolite is approximately 1000-fold more potent than CPT-11 [4]. Only 2–8% of CPT-11 is converted into Sn38 [5] with an important inter patient variability [6]. Thereby, Sn38 might be of a great use for cancer treatment. However, this drug is poorly soluble in aqueous solution and in all pharmaceutically acceptable solvents. Consequently, in order to enhance drug solubility and improve stability of Sn38, nanovectors have been developed such as micelles [7–11], liposomes [12–15] or solid lipid nanocapsules [16]. These formulations have been developed for intravenous administration but oral administration of chemotherapy is preferred by patient at 89% [17]. Indeed, oral administration improves patients' quality of life because it is practical and prevents intravenous drawbacks (catheter infection, thrombosis and extravasations).

Lipid nanocapsules (LNCs), a new generation of nanovector, have been developed in our laboratory [18]. These LNCs were obtained by a low-energy emulsification method and can be formulated in the absence of organic solvent with only generally recognized as safe (GRAS) excipients [19]. Moreover, previous studies demonstrated their capacity to increase oral bioavailability of paclitaxel [20], which can be explained by a gastrointestinal stability of particles [21] and an improvement of intestinal permeability [22]. Taking into account these advantages, the aim of the present work was to develop a formulation of Sn38-loaded LNCs. The nanocapsules were also characterized, and their ability to cross Caco-2 cell monolayers was evaluated.

2. Materials and methods

2.1. Materials

Sn38 powder was purchased from ScinoPharm Taiwan LDT (Tainan, Taiwan). Oil solubilizers and excipients were a gift from Gattefosse S.A (Saint-Priest, France), Abitec Corp. (Colombus, Ohio, USA) or Stéarinerie Dubois (Boulogne, France). Lipoid® S75-3 (soybean lecithin at 70% of phosphatidylcholine and 10% of phosphatidylethanolamine) and Solutol® HS15 (mixture of free polyethylene glycol 660 and polyethylene glycol 660 hydroxystearate) were gifts from Lipoid Gmbh (Ludwigshafen, Germany) and BASF (Ludwigshafen, Germany), respectively. NaCl was purchased from Prolabo VWR International (Fontenay-sous-Bois, France). Atenolol and propanolol powder were supplied by Sigma–Aldrich (Saint-Quentin Fallavier, France). Purified water was obtained from a MilliQ185

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System (Millipore, Paris, France). Acetonitrile, acetone, dimethylsulfoxide (DMSO), methanol, and tetrahydrofurane (THF) HPLC grade were from Sigma–Aldrich (Saint-Quentin Fallavier, France) and Carlo Erba Réactifs (Val-de-Reuil, France). Culture reagents were obtained from Sigma (St. Louis, USA) and Lonza (Verviers, Belgium).

2.2. Solubility studies

Excess amount of Sn38 was added to 1 g of various oil excipients or solvent of class 3. In order to facilitate solubilization, preparations were sonicated for 2 h, and then were filtered using a Minisart® 0.2 μm filter (Sartorius, Goettingen, Germany). Solubilization of Sn38 was determined by comparing the absorbance of filtrate to corresponding oil excipient or solvent by spectrophotometry at 385 nm.

2.3. LNC formulation

LNCs were prepared according to the original process described by Heurtault et al. [18] including numerous changes. Firstly, Sn38 was solubilized in Transcutol® HP (0.5% w/w). At this solution, Labrafil® M 1944 CS, Labrafac®, and Lipoïd® S75-3 were added, and the mixture was heated at 85 °C to solubilize Lipoïd®. After cooling, Solutol® HS15, NaCl, and basic buffer (pH = 9.8, prepared by dissolving sodium borate and anhydrous sodium carbonate in water) were added and homogenized under magnetic stirring. Basic buffer was added in order to transform free Sn38 lactone to Sn38 carboxylate. Three cycles of progressive heating and cooling between 65 and 90 °C were then performed, and at 70 °C during the last cycle, an irreversible shock was induced by dilution with 2 °C acidic buffer (pH = 2, prepared by dissolving potassium chloride and sodium hydrochloride in water). Afterward, a slow magnetic stirring was applied to the suspension of LNCs for 5 min at room temperature. A final filtration was performed on LNCs to remove potential residual components.

2.4. Conductivity measurements

The conductivity was measured using a conductimeter (Consort C561, Consort NV, Turnhout, Belgium) with a two platinum plate attachment electrodes (Fischer Bioblock Scientific, Illkirch, France). Conductivity was determined during heating and cooling, between 50 °C and 95 °C, under magnetic stirring

2.5. LNC characterization

2.5.1. Particle size and zeta potential measurements

The size, polydispersity index (PI), and charge distribution of LNCs were determined by dynamic light scattering on a Zetasizer® Nano serie DTS 1060 (Malvern Instruments S.A., Worcestershire, UK). The polydispersity index was used as a measurement of the size distribution. A small value of PI (<0.3) indicates an unimodal size distribution. Nanocapsules were diluted 1:20 (v/v) in deionised water in order to assure convenient scattered intensity on the detector.

2.5.2. LNC drug payload and encapsulation efficiency

Because of the yellow color of the Sn38, the loading was determined by spectrophotometry at 385 nm after dissolving LNCs in solvent mixture as described below using a Uvikon 922 Spectrophotometer, Bio-Tek Kontron Instruments, Saint Quentin-en-Yvelines, France. First, LNCs were filtered using a Minisart® 0.2 μm filter (Sartorius, Goettingen, Germany) in order to eliminate the residual components of the LNCs. At 500 μL of filtrate, 20 μL of chlorhydric acid 1 M were added, and a new filtration was performed using a Minisart® 0.2 μm filter. Indeed, at acidic pH, Sn38 carboxylate was transformed in lactone form which precipitated at this low pH, so after acidification, free Sn38 precipitated and was eliminated. Three samples were prepared by dissolution of 100 μl of filtrate acidic Sn38-loaded LNCs in 900 μl of 5/50/45 (v/v/v) DMSO/methanol/THF solution. Quantification was achieved by comparing the absorbance at 385 nm of Sn38 sample to a calibration curve made with unloaded LNCs and a Sn38 DMSO/methanol/THF solution. The range of linear response was 0.07–0.49 mg/ml. The lower limit of detection was 0.05 mg/ml. Samples were performed in triplicate, and the mean ± SD Sn38 payload was calculated (mg of Sn38/g of LNC dispersion). The encapsulation efficiency (%) was determined by dividing the experimental drug payload by the theoretical drug payload.

2.6. Stability of LNCs in dispersion medium

Sn38-loaded LNCs were diluted 1:200 (v/v) in phosphate-buffered saline (PBS, pH = 7.4) to reach sink conditions and placed at 37 °C at 150 rpm. A sample of 0.5 mL was removed and replaced by PBS at various times intervals. Samples were acidified and filtered using a Minisart® 0.2 μm filter (Sartorius, Goettingen, Germany) in order to remove free precipitated Sn38. Then, drug payload was determined by LC–MS/MS. Drug release was calculated by difference with the theoretical drug payload, and profiles (percent release versus time) were plotted.

2.7. In vitro stability in simulated gastrointestinal media

The stability of Sn38-loaded LNCs was measured in simulated gastric (SGF) [23] and intestinal media (FaSSIF-V2 and FeSSIF-V2) [24]. The LNCs were diluted at a final concentration of 10% v/v and incubated at 37 °C in different media. Samples were collected at times 0, 1, 2, 3, and 6 h in intestinal media. Samples were centrifuged for 5 min at 1400 rpm to precipitate the enzymes. Then, samples were acidified and filtered using a Minisart® 0.2 μm filter to remove free precipitated Sn38, and drug payload was determined by LC–MS/MS in triplicate.

2.8. LC–MS/MS analysis

Chromatography was performed using a Waters Alliance® 2695 system (Waters SA, Saint-Quentin-en-Yvelynes, France) with a Xbridge® C18 50 × 2.1 mm, 5 μm column (Waters, Milford, Ireland). The mobile phase was 10 mM ammonium acetate (pH = 3.5)/acetonitril, 65:35 (v/v) isocratically and maintained for 3 min. The flow rate was 0.2 mL/min. The total HPLC effluent was directed into a Quattro Micro® triple quadruple mass spectrometer (Waters S. A.). Ionization was achieved using turbo ion spray in positive ion mode. The mass spectrometer operated in multiple reaction monitoring (MRM) mode. The $(M-H)+m/z$ transitions for each compound were 393.1 → 293.1. A typical retention time of Sn38 was found to be 1.67 min. Quantification was achieved with QuantLynx® (Waters S.A.) by comparison of the observed peak area ratios of Sn38 samples to a calibration curve obtained under the same experimental conditions. The range of linear response was 0.2–195 ng/mL. The lower limit of detection was 0.05 ng/mL, and the lower limit of quantification was 0.2 ng/mL.

2.9. Storage stability studies

The stability of produced Sn38-loaded LNCs dispersion was evaluated after storage at 2–8 °C during 10 months. The pH, particle size distribution, zeta potential, and drug payload of sample were determined after filtration of sample using a Minisart® 0.2 μm filter (Sartorius, Goettingen, Germany).

2.10. In vitro transepithelial transport

The human colon adenocarcinoma cell line (Caco-2) was obtained from the American Type Culture Collection and used between passages 25–35. Cells were cultured in Dulbecco's modified Eagle medium (D-MEM, high glucose) supplemented with 15% (v/v) fetal bovine serum, 1% (v/v) non-essential amino acids, 1% (v/v) sodium pyruvate, and 1% antibiotic solution (1×10^4 UI/mL penicillin, 10 mg/mL streptomycin, 25 μg/mL amphothericin B) in a humidified incubator 5% CO_2/95% air atmosphere at 37 °C. Cells were plated on 75 cm^2 flask at a density of 1×10^6 cells/flask and were then harvested at 80% confluence with trypsin–EDTA and seeded onto polycarbonate membrane filters (0.4 μm pore size, 1.12 cm^2 growth area) inside Transwell® cell culture chambers (Corning Costar, Cambridge, MA) at a density of 0.1×10^6 cells/insert. The culture medium (0.5 mL per insert and 1.5 mL per well) was replaced every 2 days for the first 2 weeks and every day thereafter. After 21–23 days in culture, cell monolayers were used for the following assays.

The transports of different formulations of Sn38, i.e. free Sn38 and Sn38-loaded LNCs, were studied in the apical to basolateral direction on Caco-2 cells. The test solutions were diluted in Hanks' buffered salt solution (HBSS) at 5 μM Sn38 concentrations. The experiment was started by adding 0.5 mL of test solution at apical side and 1.5 mL of HBSS at basolateral side. Then, inserts were incubated at 37 °C. A sample of 50 μL and 150 μL was removed and replaced by HBSS at various times intervals at apical and basolateral side, respectively. Sn38 content was determined by LC–MS/MS. Apparent permeability coefficient (P_{app}), expressed in centimeters per second, was calculated according to the following equation [25,26]: $P_{app} = dQ/dt \times 1/AC_0$, where dQ/dt is the rate of drug appearance on the basolateral side ($\mu g\ s^{-1}$), C_0 is the initial concentration over the apical side ($\mu g\ mL^{-1}$), and A is the surface area of monolayer (cm^2).

Before experiments, cell monolayers were washed twice with Hank's buffered salt solution (HBSS) for 15 min at 37 °C. The transepithelial electrical resistance (TEER) of monolayers was checked before and after each experiment by using a Millicell®-ER system (Millipore Corporation, Bedford, MA). Only cell monolayers with TEER values over 250 $\Omega\ cm^2$ were used. Then, atenolol and propanolol transports were evaluated for 2 h to control paracellular and transcellular transport [27]: values of $20 \pm 0.5 \times 10^6\ cm\ s^{-1}$ and $1.0 \pm 0.2 \times 10^6\ cm\ s^{-1}$ for apparent permeability of propanolol and atenolol were accepted.

2.11. Statistical analysis

Results were expressed as mean values ± SD. A Student's *t*-test was used for statistical comparison/analysis. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Solubility studies

As shown in Table 1, Sn38 was not solubilized at 0.5% (w/w) in different oils that can be used to formulate directly lipid nanocapsules. In class 3 solvents, Sn38 was solubilized at 0.5% (w/w) only in DMSO and formic acid. However, Sn38 can be solubilized in 0.1 M NaOH and in Transcutol® HP (a co-surfactant). A solubility of 5 mg/g was estimated in Transcutol® HP.

3.2. Pre-formulation

Different formulations were tested in order to encapsulate Sn38 (Fig. 2 and Table 2). Firstly, Sn38 solubilized in DMSO or in formic acid was added to the LNCs formulation, previously developed by Heurtault et al. [18], and then, solvent was evaporated. Nevertheless, the addition of these two solvents does not allow encapsulation of Sn38 in LNCs (Table 2, formulation A).

Table 1
Solubility of Sn38 in different oils, class 3 solvents (NS: no soluble, S: Soluble).

	Solubility at 0.5% p/p
Oil solubilizer/excipient	
Alpha-tocopherol	NS
Capryol® 90	NS
Capryol® PGMC	NS
Captex® 200	NS
Captex® 500	NS
Captex® 8000	NS
Sunflower oil	NS
Labrafac® CC	NS
Decyl oleate	NS
Ethyl mysirstate	NS
Ethyl oleate	NS
Lnwitor® 380	NS
Precirol® ATO5	NS
Labrafil® M1944CS	NS
Labrasol®	NS
L.A.S®	NS
Lauroglycol® 90	NS
Lauroglycol® FCC	NS
Myritol® 318	NS
Olive oil	NS
Plurol® isostearique	NS
Plurol® diisostearique	NS
Plurol® oleique	NS
Rapeseed oil	NS
Sesame oil	NS
Stearic acid	NS
Class 3 solvent	
Acetone	NS
Anisol	NS
1-Butanol	NS
2-Butanol	NS
Ter-butylmethyl ether	NS
Dimethylsulfoxide	S
Ethanol	NS
Ethyl acetate	NS
Ethyl ether	NS
Ethyl formate	NS
Formic acid	S
Heptane	NS
Isobutyl acetate	NS
Methylisobutyl ketone	NS
1-Pentanol	NS
1-Propanol	NS
2-Propanol	NS
Tetrahydrofurane	NS

LNCs formulation was then performed after solubilization of Sn38 in NaOH. Sn38 has a lactone ring ionized in carboxylate form in alkaline solution (Fig. 1). In the presence of 0.1 N NaOH, Sn38 carboxylate is solubilized in aqueous solution but addition of acidic buffer converted Sn38 in the closed lactone ring form which is insoluble in aqueous solution [14,15]. Based on these properties, a LNC formulation was performed with Sn38 solubilized in NaOH, and in order to encapsulate Sn38 in lactone form into the oily nanocapsules core, the dilution which induced the formation of particles was performed with acidic buffer. With this method, the drug payload and encapsulation efficiency were still low (<5%), which is not acceptable for pharmaceutical production in the industry (Table 2, formulation B).

Based on the Sn38 solubility in Transcutol® HP, novel formulation approach was to add this component at the LNCs formulation. However, Transcutol® is a co-surfactant, and consequently, it cannot replace alone the component used as the oily phase of LNCs. To add Transcutol® HP on the regular process of LNCs formulation

Table 2
Composition and characterization (size, drug payload and encapsulation efficiency) of different batches of LNCs.

Formulation	*A*	*B*	*C*	*D*	*E*
Quantity					
Solvent (DMSO, acid formic) (ml)	0.5	–	–	–	–
Transcutol® HP/Sn38 (g)	–	–	0.70	0.70	0.70
Labrafil® M1944 (g)	–	–	1.00	1.00	1.00
Labrafac® (g)	1.2	1.2	–	–	0.40
Lipoid® S75 (g)	0.067	0.067	0.15	0.15	0.15
Solutol® HS15 (g)	1	1	1.00	1.00	1.00
NaCl (g)	0.073	0.073	0.10	0.10	0.10
Water (g)	1.80	–	1.80	1.38	1.38
Alkalin buffer (g)	–	1.80	–	0.42	0.42
Water at 2 °C (ml)	11.2	–	5	–	–
Acidic buffer at 2 °C (ml)	–	11.2	–	5	5
Characteristics					
Size (nm)	55	51	26	35	38 ± 2
Drug payload (mg/g)	0	>0.02	>0.04	>0.23	0.43 ± 0.06
Encapsulation efficiency (%)	0	>5	>16	>72	89 ± 10

[18], oil component had to be modified. Labrafil® M1944, a hydrophilic oil in which Transcutol® is soluble, was thus added as oily phase but a drug payload of 0.04 mg/g of suspension and an encapsulation efficiency of 16% (Table 2, formulation C) were only obtained.

This low encapsulation efficiency was related to the precipitation of Sn38 during the three cycles of heating and cooling corresponding to the phase inversion steps. To avoid this phenomenon, water was replaced by an alkaline buffer at pH 9 in order to change Sn38 into the carboxylate form which is soluble in the aqueous phase of the emulsion. At the last step of the process (dilution with addition of a large quantity of water to form LNCs), an acidic buffer was added in order to change Sn38 in its lipophilic form (lactone), thus ensuring its encapsulation in the oily core of the nanocapsules. The ratio of entrapped Sn38 prepared with this modification was 72%, and the drug payload was 0.23 mg/g of suspension (Table 2, formulation D).

Finally, Labrafac® was added in order to improve lipophilic surfactant (Lipoïd®) solubilization, which was limited with Labrafil® M1944. This last formulation has a polydispersity index <0.25, a narrow size between 36 and 40 nm, and zeta potential values were ranged from −7 to −9 mV. A drug payload of 0.43 ± 0.06 mg/g of suspension and encapsulation efficiency higher than 89% was obtained (Table 2, formulation E).

3.3. Characterization of Sn38-loaded LNCs

A final formulation was hold from Sn38 encapsulation. The conductivity evolution of this formulation was measured to characterize the formulation process. Fig. 3 shows experimental values of conductivity against temperature recorded for final formulation with mixture of Solutol®, Labrafil®, Labrafac®, Lipoid®, NaCl, NaOH, and acidic buffer. Mixture was heated between 50 °C and 95 °C. Curves can be divided into three parts. At low temperature values (<75 °C), the conductivity remained constant above 7 mS/cm indicating the presence of an oil-in-water (o/w) emulsion. When the temperature increased, a sharp decreased in the conductivity was observed, and the system appeared translucent reflecting the presence of microemulsion. This behavior was suggestive of a phase inversion, which is representative of the principle used by Heurtault et al. [18]. The temperature corresponding to the lower limit of the phase inversion zone during cooling is 70 °C. A good reproductivity of the curves was also observed during the three successive heating–cooling. So, three cycles were required in order to obtain stable phase inversion. Moreover, color change from bright yellow translucent to milk pale yellow was observed during cycling and after cooling dilution with acidic buffer. This observation characterized microemulsion with Sn38 carboxylate form and suggested that Sn38 was entrapped in lactone form in the dispersion of LNCs.

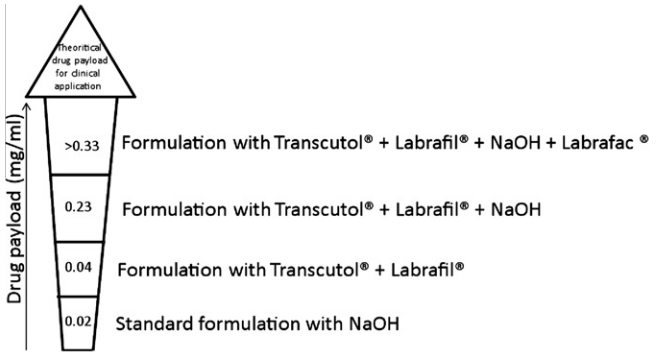

Fig. 2. Schema of enhancement of drug payload by modifying formulation parameters.

3.4. In vitro release profile

Fig. 4 reports the percentages of Sn38 released from Sn38-loaded LNCs into PBS buffer versus time. The initial burst release was prominent during the first day of release. After this burst release, a constant slow Sn38 release about 8% was observed after 3 days, showing a typical sustained and prolonged drug release.

3.5. Storage stability

Stability of particles was assessed for 10 months by storing the suspension at 2–8 °C, in its native state. As shown in Table 3, Sn38-loaded LNCs were physically stable at 2–8 °C for at least 10 months. No significant changes in mean vesicle size, pH, and

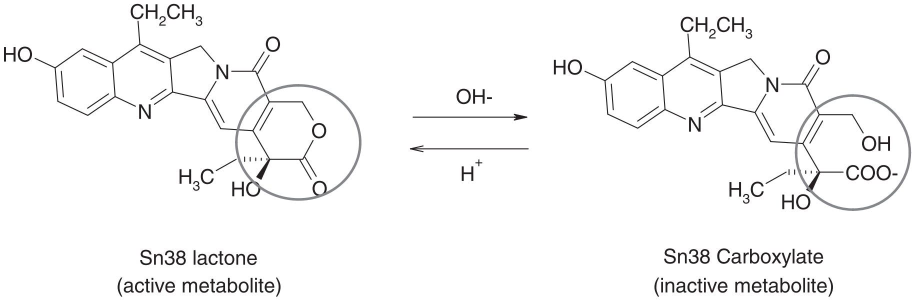

Fig. 1. pH-dependent equilibrium of Sn38.

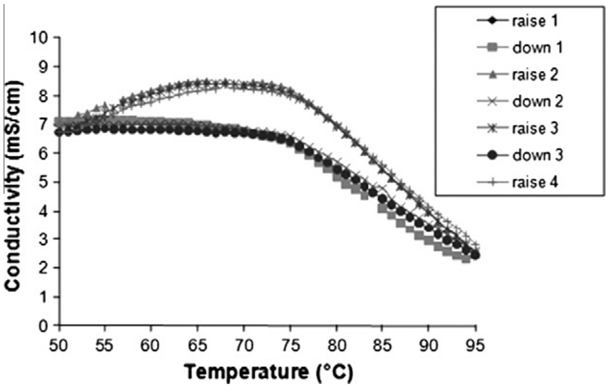

Fig. 3. Evolution of the conductivity as a function of the temperature. Three cycles ranging from 50 to 95 °C were applied.

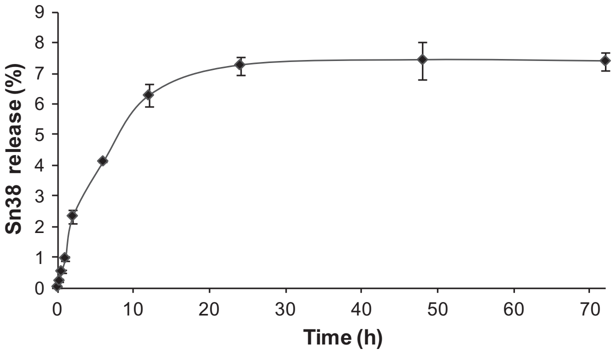

Fig. 4. Sn38 release profile from LNCs in PBS ($n = 3$; data are shown as mean ± SD).

zeta potential were observed. However, a decrease in the drug payload was observed, and the release of Sn38 was evaluated about 60%, after 10 months.

3.6. In vitro stability in gastrointestinal fluids

In view of an oral administration of this formulation, *in vitro* stability in different gastrointestinal fluids was performed. Firstly, in the gastric fluid characterized by a pH of 1.2 and the presence of pepsin, a digestive protease, a release of 20% of the initial amount of encapsulated Sn38 was measured after 3 h (Fig. 5A). Secondly, stability of Sn38-LNCs was assayed in two different intestinal fluid simulated media. FaSSIF-V2 and FeSSIF-V2 are two recent biorelevant dissolution media developed by Jantratid et al. [24,28]. These media reflect the influence of digestion processes better than media typically used [23,29]. Particularly, FeSSIF-V2 contains lipolytic product and pancreatin which requires bile salt in order to be functional in accordance with *in vivo* data. After 6 h, Sn38 remains encapsulated in LNCs (Fig. 5B) in both media (less than 10% are release). Consequently, Sn38-loaded LNCs could be administered in a pre- or post-prandial state.

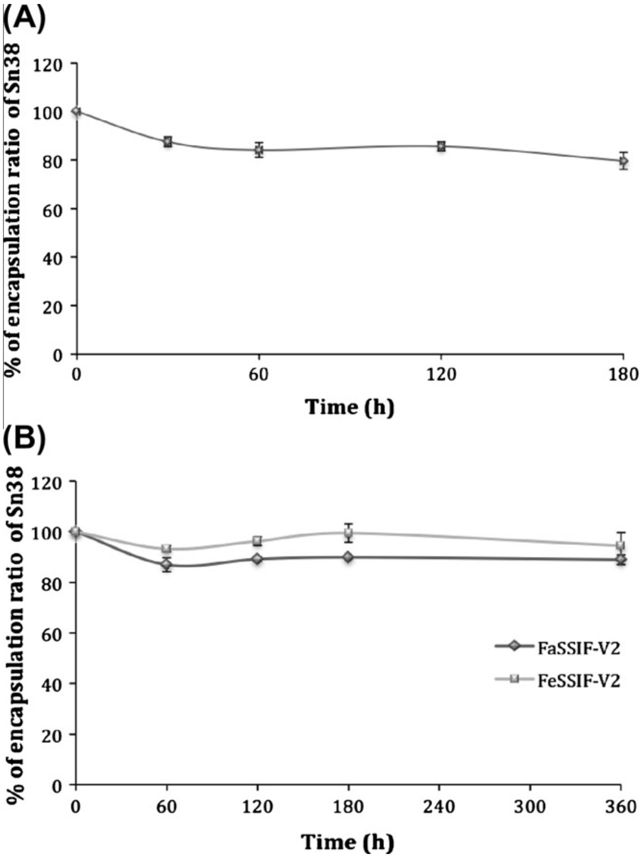

Fig. 5. Percentage of release of Sn38 following incubation in simulated gastric fluid (SGF) (A), simulated intestinal fluid in the fasted state (FaSSIF-V2), and simulated intestinal fluid in a fed state (FeSSIF-V2) (B) ($n = 3$; data are shown as mean ± SD).

3.7. Cellular transport and uptake studies across Caco-2 cell monolayers

The permeability of Sn38 was also evaluated across Caco-2 cell monolayers during 6 h. During experiment, the transepithelial resistance (TEER) was measured. TEER values were superior at 250 Ω cm^2, and no significant modifications were observed in the presence of Sn38-LNCs after 6 h (data not shown). This result indicated that Sn38-loaded LNCs did not affect Caco-2 cell monolayers. The apparent permeability was also calculated (Fig. 6A). The results showed that the apparent permeability of free Sn38 was $0.31 \pm 0.02 \times 10^6$ cm s^{-1} and remained stable during 6 h. As Sn38 was encapsulated in LNCs, an enhancement of transport of Sn38 was observed. At 2 h, the apparent permeability was $1.63 \pm 0.56 \times 10^6$ cm s^{-1} and increased to $5.69 \pm 0.87 \times 10^6$ cm s^{-1} at 6 h. In addition, an increase by a factor 2 of absolute intracellular amounts of Sn38 was observed (Fig. 6B) for Sn38-LNCs. This result indicated that LNCs noticeably improved the *in vitro* intestinal transport of Sn38.

Table 3
Stability data of Sn38-loaded LNCs stored at 2–8 °C (size, polydispersity, zeta potential, pH and drug payload) ($n = 6$).

Times (month)	Initial	0.5	1	3	10
Size Sn38-LNCs (nm)	38 ± 2	39 ± 1	39 ± 1	40 ± 1	41 ± 1
Polydispersity index Sn38-LNCs	0.141 ± 0.042	0.147 ± 0.055	0.141 ± 0.037	0.135 ± 0.037	0.127 ± 0.017
Zeta potential Sn38-LNCs (mV)	−7.7 ± 1.0	−7.6 ± 0.3	−8.5 ± 0.4	−8.6 ± 0.7	−9.0 ± 2.5
pH Sn38-LNCs	7.3 ± 0.1	7.6 ± 0.08	7.4 ± 0.03	7.5 ± 0.03	–
Drug payload (mg/g of dispersion)	0.43 ± 0.06	0.41 ± 0.08	0.34 ± 0.04	0.33 ± 0.06	0.19 ± 0.02

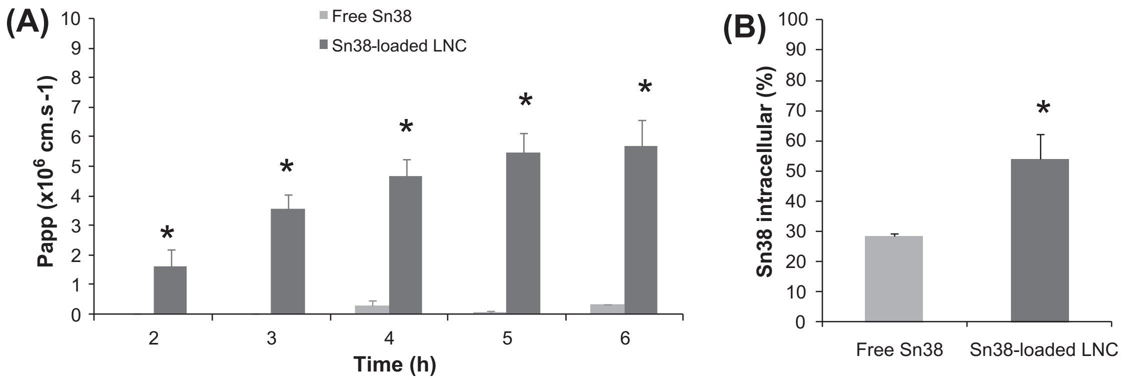

Fig. 6. Variation of apparent permeability coefficients and intracellular uptake of Sn38 after 6 h of incubation with free Sn38 and Sn38-loaded LNCs. (A) Variation of apparent permeability of Sn38 for 6 h. (B) Percentage value indicates the intracellular quantities of Sn38 (%), initial concentration of Sn38 was considered as 100% of Sn38 intracellular ($n = 4$; data are showed as mean ± SD, "*" is displayed for series significant different from control (free Sn38), $P < 0.05$ – Student's *t*-test).

4. Discussion

To date, different intravenous formulations of Sn38 were developed: liposomes [14,15] and polymeric micelles [7,8,11], and recently, a poly(amidoamine) (PAMAM) dendrimers were evaluated to improve the oral bioavailability of Sn38 [30]. To formulate dendrimers or micelles, Sn38 was covalently complexed with polymer, and consequently, it was not protect against eventual degradation along the gastrointestinal tract. To formulate liposome, Sn38 was dissolved in mixture of methanol and chloroform and added to different lipid films. Consequently, in this paper, we tried to prepare nanoparticles of Sn38 without solvent. To formulate nanovectors such as lipid nanocapsules, Sn38 has to be solubilized preferentially in the components of formulation [31–34] (oil, Solutol®, lipoïd®) or in co-surfactants, which can be added to the preparation [20]. Results of this study show that among oils and solvents tested, none can solubilize Sn38 (Table 1) and permit to encapsulate it in LNCs.

Finally, a co-surfactant, Transcutol®HP, permits to solubilize Sn38. Transcutol® was used in some emulsions or microemulsions [35–37] as a co-surfactant; consequently, modifications on the formulation process of LNCs [18] were required to add it. Several Sn38-LNCs prototype formulations were investigated in terms of oily phase and aqueous phase. Upon further optimization (Table 2), a lead formulation of Sn38-loaded LNCs was developed and characterized.

The concomitant presence of Transcutol®HP with Labrafil® M1944/Labrafac® (oily phase), Solutol®, Lipoid® (surfactants), and NaOH (aqueous phase) does not modify the inversion phase (Fig. 3), which is representative of the principle used by Heurtault et al. [18]. The conductivity measurement characterized the phase inversion zone, which described the inversion from O/W emulsion to W/O emulsion. Nevertheless, at low temperature (Fig. 3), the emulsion was not characterized by a high conductivity (as 35 mS/cm observed by Heurtault et al.) while characteristic of the aqueous continuous phase of an O/W emulsion; thus, a w/O/W emulsion system was suggested [38]. Then, when temperature was increased, a rapid conductivity decrease was observed, which is characteristic of a W/O emulsion. The phase inversion zone (PIZ) began at 72 °C, and consequently, the irreversible shock was performed around 70 °C (2 °C from the beginning of the PIZ in the w/O/W emulsion). Furthermore, results suggest Sn38 entrapment in lactone form due to the milk pale yellow color of the LNCs dispersion in comparison with the bright yellow color observed during the three heating–cooling cycles. Indeed, during formulation process, Sn38 was in carboxylate form in order to limit its precipitation in aqueous phase. The transformation of Sn38 in lactone form is important to keep the inhibitor activity on topoisomerase I [39,40].

This Sn38-LNCs formulation has a polydispersity index <0.25, which demonstrates the monodispersity of the preparation, and the average size was 38 ± 2 nm. In a recent work [22], our team demonstrated that a size inferior to 100 nm was interesting in order to increase transport by endocytosis across the intestinal barrier. In consequence, this size is an advantage to consider an oral administration of Sn38-LNCs.

After 3 days into PBS buffer, the percentage of Sn38 released was about 8% and reached a plateau. This release profile suggests that Sn38 remained associated with LNCs during the study. This result was superior to Sn38 released from liposomal formulation (1.9% Sn38 released over 120 h) [15], but is still too low. A better profile of release was observed with micelles (>54% at 24 h) [7] with which a superior antitumor activity than CPT-11 was demonstrated. Consequently, this incomplete release of Sn38 with LNCs could be a limit to an antitumor activity if it is confirmed *in vivo*. It is worth to note that the *in vitro* conditions of the release studies were not favorable because of the low solubility of Sn38 in aqueous media. These conditions were chosen to compare the new formulations with the other described in the literature. *In vivo*, the presence of proteins should help the release of Sn38.

Finally, when the LNC suspension was stored at 4 °C, no significant size variation was observed indicating no destabilization for LNCs. Because nanoparticles were analyzed after filtration, one cannot determine whether aggregate over 220 nm is formed during the aging of the formulations. However, a release of Sn38 was observed. This release could be explained by the external pH of aqueous phase (pH 7.4). At this pH, a non-negligible fraction of Sn38 was present in the inactive carboxylate form (approximately 60% [15]), which displays a significant aqueous solubility [41,42], and consequently, might be released from the lipid nanocapsules. To overcome this problem, lyophilization of Sn38-LNCs could be carried out. Indeed, Zhang et al. [15] demonstrated no significant change in drug entrapment of lyophilized liposome-based Sn38 after 6 months and a stability for diluted liposomes over 8 h. Moreover, classical LNCs could be freeze-dried in presence of trehalose without any problem [43].

For patient's convenience, the oral route is a must. Nevertheless, oral absorption of drug is limited by various physiological barriers [44]. For anticancer drug, many solutions have been sought to obtain oral formulations, and nanometric-sized drug delivery systems may provide an alternative solution. The administration of nanocarriers via oral route is however difficult because many biopharmaceutical parameters have to be considered in order to obtain a good efficacy/safety ratio. In view of an oral administration of Sn38-LNCs, some properties of this formulation have to be studied. The first barrier to overcome after oral administration is constituted by the physicochemical environment of the gastrointestinal tract. Even if a previous study demonstrated that LNCs were stable in gastrointestinal tract [21], we investigated the stability of these Sn38-LNCs which differed from classical LNCs. Firstly, in the gastric fluid, a release of Sn38 from LNCs was observed after 3 h (Fig. 5A). Khohatkar et al. [30] who demonstrated the potential of poly(amidoamine) (PAMAM) dendrimers to improve the oral bioavailability of Sn38 noticed the lack of PAMAM to be stable in the gastric environment. Indeed, acidic pH is responsible for the release of Sn38 from the complex because complexes were formed by ionic interaction of the positive charges of surface amine group of dendrimers with carboxylate Sn38 molecules. In case of LNCs, we supposed that the Sn38 lactone form was encapsulated into the lipid core of LNCs and was consequently protected by the shell composed by Solutol®. But, some Sn38 molecules could be present on the shell of LNCs and thus, could be released in the acidic environment. Moreover, in fasted and fed simulated intestinal fluids, Sn38 remains encapsulated in LNCs (Fig. 6B). In accordance with these results, a pre-prandial state administration could be preferable to limit residence time in the stomach. Sn38-LNCs could be also considered as a way to protect Sn38 in the gastrointestinal tract.

The low systemic availability of drug after oral administration is mostly attributed to limited drug absorption across the intestinal epithelium. Consequently, the permeability of Sn38-LNCs was studied across Caco-2 cell monolayers. Caco-2 cell model is a well-established model used to study the intestinal permeability of drugs [25,45]. After differentiation, Caco-2 cells formed a monolayer of polarized cells which present tight junctions and active transporters such as the P-glycoprotein [25,26,45,46]. A noticeably enhancement of Sn38 transport across Caco-2 cell monolayers (Fig. 6) was demonstrated with Sn38-LNCs, and an increase by a factor 2 of absolute intracellular amounts of Sn38 was observed (Fig. 6B). Uptake of Sn38 was previously demonstrated [47–49] but it was variable and responsible of severe diarrhea observed in patients treated with CPT-11. Thus, encapsulation in LNCs could solve these two limitations. Furthermore, Sn38 is actively transported by P-glycoprotein [47,50,51]. So, enhancement of Sn38 uptake could be explained by the capacity of LNCs to inhibit the P-gp. Indeed, our group demonstrated that LNCs could inhibit the activity of P-gp [33,52] due to the presence of Solutol® in their shell [53,54].

Consequently, the present study shows that Sn38-LNCs could be a good candidate for oral administration, as these particles are stable in gastrointestinal media and provide an enhancement of Sn38 permeability across a model of intestinal barrier. In fact, an improved permeability could be a first step to obtain more predictable blood concentration of the drug and the desired pharmacological effect.

5. Conclusion

In conclusion, Sn38-loaded LNCs, using Transcutol® HP as a co-surfactant, Labrafil® M 1944 CS as part of the oily phase, and the pH-dependent solubility of Sn38, were obtained by the phase inversion temperature method with a size and drug payload potentially compatible with clinical use. Our data provide also evidence that these nanocarriers are promising for oral delivery of Sn38. The next objective is to evaluate the pharmacokinetic and the therapeutic effect of these LNCs, in particular, after oral administration.

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