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Development and *in vitro* evaluation of a novel lipid nanocapsule formulation of etoposide

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ABSTRACT

Small cell lung cancer (SCLC) is the most aggressive carcinoma in thoracic oncology, unfortunately, despite chemotherapy, relapse is constant. The effect of etoposide, a major drug used against SCLC, can potentially be enhanced after its encapsulation in nanocarriers. The aim of this study was to use the technology of lipid nanocapsules (LNCs) to obtain nanocarriers with drug loadings compatible with clinical use and with an industrial process. Solubility studies with different co-solvent were first performed, then several process were developed to obtain LNCs. LNCs were then characterized (size, zeta potential, and drug loading). The best formulation called Ω-LNCs had a size of 54.1 ± 2.0 nm and a zeta potential of −5.8 ± 3.5 mV and a etoposide drug loading of 5.7 ± 0.3 mg/g. The characteristics of this formulation were maintained after freeze drying and after a 15× scale-up. Release studies in a media mimicking plasma composition showed that 40% of the drug was released from the LNCs after 48 h. Moreover the activity of etoposide after encapsulation was enhanced on H209 cells, IC50 was 100 μM and 2.5 μM for etoposide and etoposide LNCs respectively. Unfortunately the formulation failed to be more cytotoxic than etoposide alone on H69AR cells that are resistant to etoposide. This study showed that is was possible to obtain a new etoposide nanocarrier without the use of organic solvent, that the process is suitable for scale-up and freeze drying and finally that etoposide activity is maintained which is very promising for future treatment of SCLC.

1. Introduction

Small cell lung cancer (SCLC) is the most aggressive carcinoma in thoracic oncology (van Meerbeeck et al., 2011). Because of the short doubling time and high intrinsic spreading capacity of this cancer, nearly all patients have metastatic disease at diagnosis explaining why chemotherapy is the main therapy. Although a high chemosensibility in chemo naïve patients, relapse is constant.

Etoposide (4′-demethyl-epipodophyllotoxin) remains one of the pivotal drug against SCLC (Hainsworth and Greco, 1995; You et al., 2008). The action of this molecule is to inhibit human topoisomerase II which leads to apoptosis of tumor cells (Beauchesne et al., 1999). In its commercial form, it can be combined with alcohol, and surfactant like polysorbate 80 or Cremophor EL (polyethoxylated castor oil) and diluted in physiological salt solution. These additives display cell toxicity and modify the pharmacokinetics of the drug (Ellis et al., 1996; Jelinek and Klocking, 1998). Two formulations of etoposide are currently used: capsules for oral administration and solution for intravenous administration. Encapsulation of the drug in colloidal carriers could allow a higher concentration of etoposide in primary tumor and metastatis due to the enhanced retention and permeation (EPR) effect and theoretically improve safety and efficiency of the drug (Huynh et al., 2010).

Up to now, different nanoparticles containing etoposide were formulated mainly in polymeric nanospheres (Callewaert et al., 2012; Gaucher et al., 2007; Kilicay et al., 2011; Poreba et al., 2011; Yadav et al., 2011) or solid lipid nanoparticles (Khajavinia et al., 2012; Patlolla and Vobalaboina, 2008; Reddy et al., 2006; Zhang et al., 2011). Others nanoparticles such as liposomal formulations (Jinturkar et al., 2012), micelles (Mohanty et al., 2010; Varshosaz et al., 2012) or dendrimers (Sideratou et al., 2010) were also evaluated. Unfortunately, most of these particles has major drawbacks such as presence of organic solvent or toxic compounds

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preventing their exploitation in clinical use. On the contrary solvent free lipid nanocapsules (LNCs) have shown a very good toxicological profile after intravenous infusion and are produced only with GMO free and generally recognized as safe (GRAS) excipients (Hureaux et al., 2009, 2010).

The aim of this study was to develop solvent-free lipid nanocapsules able to entrap etoposide for human use. Then the LNCs formulated were characterized, the release was studied in a biomimetic environment and tested on NCI-H209 and H69AR, cell lines of SCLC sensible and resistant to etoposide (Hillgenberg et al., 1999; Mirski et al., 1987), respectively.

2. Materials and methods

2.1. Materials

Etoposide powder was purchased from Ascent Scientific (Bristol, Great-Britain). Oil solubilizers and excipients were provided by Gattefosse S.A. (Saint-Priest, France), or Abitec Corp. (Colombus, USA), or purchased from Sigma–Aldrich (Saint-Quentin Fallavier, France) or Fluka (Buchs, Switzerland). Lipoïd® S75-3 (soybean lecithin at 70% of phosphatidylcholine and 10% phospatidylethanolamine) and Solutol® HS15 (mixture of free polyethylene glycol 660 and polyethylene glycol 660 hydrostearate) were a gift from Lipoïd Gmbh (Ludwigshafen, Germany) and BASF (Ludwigshafen, Germany), respectively. NaCl was purchased from Prolabo VWR International (Fontenay-sous-Bois, France). Purified water was obtained from a MilliQ185 System (Millipore, Paris, France). Methanol HPLC grade was purchased from Fisher Scientific (Loughborough, Great-Britain). Reagents for activating complement test were purchased from Eurobio (Courtaboeuf, France), Merck (Fontenay-sous-Bois, France), Sigma–Aldrich, Fluka (Buchs, Switzerland) and the Etablissement Français du Sang (Nantes, France). Culture reagents were obtained from Lonza (Verviers, Belgium).

2.2. Solubility studies

In order to determine the oils or excipients which could solubilize etoposide, a screening study was performed. This task was accomplished by introducing 5 mg of etoposide in different tubes containing 1 g of each oil or excipients. After 24 h at room temperature, tubes were observed visually in order to evaluate the etoposide solubility. Then, the samples were introduced in a water bath Julabo SW22 stirred at 125 rpm during 24 h at 60 °C and the etoposide solubility was again evaluated at this temperature. If the powder had visually disappeared, the minimum solubility of etoposide in the compound was considered to be 0.5% (w/w).

The maximum solubility of etoposide in oil or excipient was finally determined by high performance liquid chromatography (HPLC), following an adaptation of a method previously described (Shirazi et al., 2001). The apparatus was composed by injector (Waters® 717plus), pump (Waters® 660 E), detector (Waters® 2487), controller (Waters® 600), software: Millenium 32 version 3.2 (Waters®, Saint Quentin-en-Yvelines, France). The column used was a Sunfire® C18 5 μm 4.6 ∗ 150 mm from Waters®. Injected volume and run time were respectively 20 μL and 8 min. The mobile phase was composed of 70% methanol plus 30% purified water, the flow rate was 1 mL/min and the detection wavelength was 228 nm. For this experiment, an excess of etoposide was introduced in hemolysis tubes. Then, the tubes were placed in a water bath Julabo SW22 at 60 °C, stirred at 125 rpm during 24 h and supernatant were then collected and filtered through Acrodisc® 13 mm filters from Pall Corporation® (Ann Arbor, USA). Finally, the filtrate was diluted 10,000 fold to obtain a concentration of etoposide in the range where it was linear with respect to the area under the curve (between 0.25 and 12 μg/mL, R^2 = 0.999, maximum deviation below 10%).

2.3. LNC formulation

2.3.1. LNC formulation without etoposide

LNCs were obtained according to the patent filed by Heurtault et al. (2001) and based on the phase-inversion process (Heurtault et al., 2002). Indeed, the PEG-chains of the Solutol® HS15, which is one of the surfactant of the LNCs, dehydrate following an increase of temperature. This phenomenon leads to lowering the HLB balance and induce the phase inversion to water in oil emulsion from oil in water emulsion.

Based on results of the solubility study and works of Roger et al. (2011), a mixture of Labrafac® CC, Labrafil® M1944CS and Transcutol® HP (0.4 g, 0.12 g, and 0.4 g respectively) was chosen as the oily phase. Then, these compounds were mixed with 150 mg of Lipoïd® S75-3, and heated by an IKA RCT Classic hot plate at 82 °C with agitation to 1200 rpm maintained until complete solubilization of this compound. Then, once the ambient temperature of the mixture was recovered, the other compounds of the formulation i.e., water, NaCl and Solutol HS15 (1.8 g, 0.1 g, and 1 g respectively) were introduced. After that, the heating cycles were performed between 60 and 90 °C with a gradient of 4 °C/min while the system was quenched at 70 °C by adding 5 mL of 0 °C deionised water. Finally, the agitation of 1200 rpm provided during every steps of formulation was maintained until the solution reached the room temperature.

2.3.2. Formulation of LNCs loaded with etoposide

In a first step, etoposide was solubilized in Transcutol® HP. Then, LNCs were synthesized with etoposide using two distinct ways. In the first way, oil solubilizing etoposide was placed in contact with other compounds and the heating cycles were performed (called Ref-LNCs further in text). In the second way, the heating cycles were operated with all compounds, except Transcutol® HP and etoposide and then Transcutol® HP solubilizing etoposide was introduced at the beginning of the last cooling step as patented previously by our group (Benoit et al, 2010).

2.3.3. Formulation of LNCs without Lipoïd® S75-3

For the formulation of LNCs without Lipoïd® S75-3 (called Ω-LNCs further in text), the percentage of each component of the oily core were kept the same but the mass had to be decreased in order to compensate the lack of this surfactant, and thus, keeping size of LNCs close to 50 nm. So, the oily mix was composed by 0.3 g of Labrafac® CC, 0.9 g of Labrafil® M1944CS and 0.3 g of Transcutol® HP.

2.4. Characterization of LNCs

LNC size distribution was measured by dynamic light scattering (DLS) and zeta potential values were assessed using a Zetasizer Nano ZS from Malvern (Orsay, France). The helium–neon laser, 4 mW, operates at 663 nm with the scatter angle fixed at 173 °C and the temperature fixed at 25 °C. Measurements were performed three times for each experimental point. Size diameter corresponds to intensity of the signal.

2.5. Determination of encapsulation efficiency and drug loading

The encapsulation efficiency determination of the etoposide was carried out by HPLC, (same experimental conditions that are described above in text). LNCs were separated from supernatant using Nanosep® Omega 30 kD microcentrifuge filters (Pall Corporation, Ann Arbor, USA) and etoposide was measured in the

supernatant. The encapsulation efficiency was determined by dividing the experimental drug loading by the theoretical drug loading.

2.6. Study of release

The determination of the release kinetics of etoposide was studied ($n = 3$) in an artificial biomimetic environment. It consisted in placing dialysis tubes (which cut-off was 50 kD) containing 16 mg of etoposide encapsulated in LNCs. Each tube was placed in 300 mL of aqueous medium containing 60 g/L of bovine serum albumin (BSA), 9 g/L of NaCl and buffered to pH 7.4 with NaOH 1 mol/L. The beakers containing the dialysis tubes were then placed in a water bath at 37 °C stirred at 50 rpm. Then, the continuous medium to the dialysis tubes was collected at 15, 30, 60, 90, 120, 240, 360 min, 18, 24, 45.5 and 72 h. In order to precipitate the protein and to determine the etoposide content, 200 μL of the continuous medium were placed in 1800 μL of methanol and were centrifuged at 10,000 rpm for 10 min. The supernatant was then assayed by HPLC (same experimental conditions that are described above in text but the linearity zone was between 0.4 and 8 μg/mL, $R^2 = 0.999$, and the maximum deviation was always found below 20%).

2.7. Hemolytic complement activation

Hemolytic complement activation tests were performed according to a protocol adapted to nanoparticles and described previously (Vonarbourg et al., 2006). Briefly, the LNCs are associated with the complement proteins which become activated and lead to the destruction of erythrocytes. The absorbance determined with the Multiskan Ascent (Labsystems SA, Cergy-pontoise, France) was then correlated with the activation percentage.

2.8. Cells experiments

2.8.1. Tumor cell culture

SCLC lines H209 and H69AR were obtained from ATCC (LGC, Molsheim, France). The cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2 in Roswell Park Memorial Institute medium (RPMI) with L-glutamine (Lonza) supplied with 20% foetal bovine serum (FBS), 1% Na Pyruvate, HEPES 0.01 M and 1% antibiotic and antimycotic solution (Sigma).

2.8.2. In vitro cell viability

H209 or H69AR cells were first plated at 4.10^4 cells/cm^2 on 96-well plates for 48 h in RPMI containing 10% FBS, 1% Na Pyruvate, HEPES 0.01 M and 1% antibiotic/antimitotic and then treated with increasing concentrations of various etoposide preparations (0.01–100 μmol/L). To test the impact of the drug alone (non-encapsulated) on cells, methanol was used to solubilize the drug. The drug in solution was prepared at a concentration of 0.1 M and a dilution of 1:1000 in culture medium was realized to obtain the higher concentration tested on cells (100 μmol/L). Blank LNCs were tested as controls and with the same excipient concentration than that needed for etoposide ones. The cells were incubated at 37 °C/5% CO_2 for 72 h. Cell viability was determined by CyQUANT® Cell Proliferation assay (Invitrogen, Carlsbad, CA), following the manufacturer's instructions. Briefly, 200 μL of cell-lysis buffer solution diluted 20-fold in distilled water and including CyQUANT® (diluted 400-fold into the 1× cell-lysis buffer) was added to each well, and the plates were incubated at room temperature for 5 min, protected from light.

Then, 100 μL of each sample was transferred into a microplate and the fluorescence was determined using 485 nm for the excitation and 515 nm for the emission wavelengths using a multiwell-scanning spectrophotometer Fluoroscan Ascent FL (Labsystems SA, Cergy-pontoise, France). Three independent repetition experiments were conducted, each with at least 3 repeated samples.

2.9. Freeze-drying of etoposide free LNCs

The storage test of etoposide free LNCs were carried out by freeze-drying with a freeze-dryer Lyovac Steris GT2 (Steris, Germany), to a pump 2005 C1 from Alcatel (Annecy, France) and a cryostat CC 505 from Huber (Barr, France).

The LNCs were freeze-dried in the presence and absence of trehalose, as cryoprotectant. When a cryoprotectant was added, the mass of trehalose was equal to the mass of dry LNCs. In each case, the mass of freeze-dried solution was 1.5 g.

2.10. Scale-up assay

The scale-up of LNCs was performed on the formulation whose characteristics seem to fit the problems of the subject. The weight of all compounds was multiplied by 15-fold.

2.11. Statistics

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

Several oily excipients were evaluated at 0.5% w/w concentration to room temperature and 60 °C. Among the 27 oils or excipients tested, only nine have concluded to a solubility of etoposide due to a partial or complete disappearance of the powder initially introduced. The results of this experiment are recorded in Table 1.

The maximum solubility of etoposide has been determined by HPLC for Transcutol®HP (74.85 mg/mL), Tween®80 (39.12 mg/mL), Capmul®907P (8.961 mg/mL) and Labrafac® CC (0.074 mg/mL).

Among the oily excipients evaluated, Transcutol HP® showed the better etoposide solubility. Moreover, it was the only product able to solubilize etoposide at room temperature.

3.2. Formulation of LNCs

In order to obtain a nanocapsule diameter close to 50 nm with a PDI inferior to 0.2, the oily core of the nanocapsules was composed by 20% Labrafac® CC, 60% Labrafil® M1944CS and 20% Transcutol® HP. These conditions allowed the encapsulation of etoposide with a medium and variable encapsulation efficiency (Table 2).

3.3. Optimization of formulation

3.3.1. Composition of the oily core

Transcutol® HP was the only component of the oily core able to solubilize etoposide. It was thus sought to increase its percentage in the lipid nanocapsules. Several formulations of LNC without etoposide have been performed, characterized in terms of stability, size diameter and polydispersity index. A ternary diagram was built with the three components (Labrafac® CC, Labrafil® M1944CS and Transcutol® HP) of the oily core (Fig. 1).

The ternary diagram showed a zone delimited by green dots where stable LNCs could be obtained (size stable along 7 days and PDI remained <0.2). In this area, the percentage of transcutol® HP in the core of LNCs could be doubled. Unfortunately, a

Table 1
Solubility of etoposide in different oils or excipients, S: solubility observed, NS: no solubility observed.

Oil solubilizers or excipients	Solubility at room temperature	Solubility at 60 °C
Capmul 907P®	NS	S
Capmul MCM-EP®	NS	S
Capmul GMO-50®	NS	S
Captex 200P®	NS	NS
Captex 225®	NS	NS
Captex 350®	NS	NS
Captex GTO®	NS	NS
Captex 300®	NS	NS
Castor oil	NS	NS
Coconut oil	NS	NS
Cod liver oil	NS	NS
Corn oil	NS	NS
Ethyl oleate	NS	NS
Labrafac CC®	NS	S
Labrafac CC® + 2% (w/w) cholesterol	NS	NS
Labrafil M1944 CS®	NS	NS
Laurygol 90®	NS	S
Maisine 35-1®	NS	NS
Oleic plurol	NS	NS
Safflower oil	NS	NS
Sesame oil	NS	NS
Solutol HS15®	NS	S
Span 80®	NS	NS
Transcutol HP®	S	S
Tween 80®	NS	S
Vitamine E®	NS	NS
Vitamine E TPGS®	NS	S

destabilization of the LNCs has been observed after encapsulation of etoposide when the core was composed of more than 20% Transcutol® HP. The formulation with 40% of Transcutol® HP (S green dot – Fig. 1) even showed a total release of etoposide after 7 days (data not shown).

3.3.2. Influence of Lipoïd® S75-3

Lipoïd® S75-3, a soybean lecithin, acts as surfactant in the formulation but its solubilization is difficult and can explain difference in encapsulation results between batches. Thus, LNCs formulated in absence of this surfactant were evaluated (Table 3). If the percentage of each component of the oily core remained the same, their amount was decreased in order to obtain an identical diameter of the nanocapsules.

The drug loading slightly decreased in the formulation of LNCs without Lipoïd® S75-3, due to the decrease of Transcutol HP in the formulation, but the encapsulation efficiency was more reproducible and the polydispersity of LNCs was lower. The zeta potential increased. In the following study, LNCs with and without Lipoïd® S75-3 were studied jointly.

3.3.3. Transcutol® HP addition

As performed classically, the active molecule was introduced into the oily core prior to the completion of three heating cycles of the solution. However, an original modification of the LNC formulation (Benoit et al., 2010), allowed to introduce the drug just before the quenching. LNCs with or without Lipoïd® S75-3 were formulated to introduce Transcutol® HP solubilizing etoposide at the beginning of cooling of the last heating (Table 4).

No major difference was observed between the formulation studied even if the addition of Transcutol® HP solubilizing etoposide during the last cooling has improved the encapsulation efficiency of etoposide for each of the formulation containing Lipoïd® S75-3 ($P < 0.05$).

3.3.4. Freeze-drying study

Before any clinical study, it will be necessary to assess the ability of LNCs loaded with etoposide to be lyophilized. Based on previous works (Dulieu and Bazile, 2005), freeze-drying tests was performed on formulations non-loaded with etoposide for safety reasons (Table 5) in the presence or absence of trehalose (as cryoprotectant). Two formulations with or without Lipoïd® S75-3 (called Ref and Ω, respectively) were evaluated.

Freeze-drying of formulation with Lipoïd® S75-3 showed an increase of the LNCs diameter and polydispersity index, even in presence of trehalose as cryoprotectant. In contrast, nanoparticles formulated without Lipoid® S75-3 could be lyophilized without change of their initial characteristics. Based on this last result, only Ω-type LNCs (i.e., without Lipoïd® S75-3) were studied in following

Table 2
Characterization of blank and etoposide loaded LNCs ($n = 3$ for each batch of LNCs).

	Size (nm)	PDI	Zeta potential (mV)	Encapsulation efficiency (%)	Drug loading (mg/g)
Blank LNCs	46.8 ± 2.9	0.089 ± 0.050	−12.06 ± 0.09	–	–
Etoposide loaded LNCs	49.2 ± 4.1	0.123 ± 0.039	−20.26 ± 3.17	56.7 ± 21.3	6.5 ± 3.0

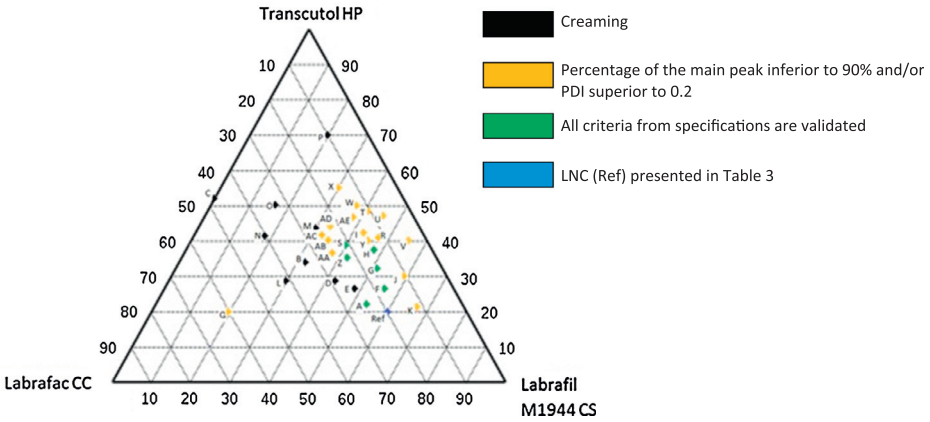

Fig. 1. Ternary diagram allowing the determination of the area of acceptable LNCs (in green): main size peak >90% and/or PDI <0.2. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 3
Characteristics of LNCs obtained with or without Lipoid® S75-3.

	Lipoid S75-3 (mg)	Size (nm)	PDI	Zeta potential (mV)	Encapsulation efficiency (%)	Drug loading (mg/g)
LNC with lipoid	14.8	49.2 ± 4.1	0.123 ± 0.039	−20.26 ± 3.17	56.7 ± 21.3	6.5 ± 3.0
LNC without lipoid	0	52.7 ± 0.2	0.070 ± 0.001	−2.24 ± 0.73	77.9 ± 2.5	5.4 ± 0.7

Table 4
Characteristics of LNCs obtained with or without Lipoid® S75-3 for 2 different protocols of Transcutol® HP adding: at the beginning of the three thermal cycles or during the last cooling step.

	Transcutol® HP adding time	Size (nm)	PDI	Zeta potential (mV)	Encapsulation efficiency (%)	Drug loading (mg/g)
LNC with lipoid	Beginning	49.2 ± 4.1	0.123 ± 0.039	−20.26 ± 3.17	56.7 ± 21.3	6.5 ± 3.0
	Last cooling	50.8 ± 3.5	0.140 ± 0.057	−16.62 ± 2.01	74.0 ± 1.6	5.8 ± 0.8
LNC without lipoid	Beginning	52.7 ± 0.2	0.070 ± 0.001	−2.24 ± 0.73	77.9 ± 2.5	5.4 ± 0.7
	Last cooling	54.1 ± 2.0	0.100 ± 0.020	−5.81 ± 3.53	75.8 ± 3.3	5.7 ± 0.3

Table 5
Characteristics of LNCs obtained after different freeze drying conditions.

Formulation	With 14.8 mg lipoid (Ref)			Without lipoid (Ω)		
Status	Before freeze drying (n = 3)	After freeze drying with trehalose (n = 1)	After free drying without trehalose (n = 1)	Before freeze drying (n = 3)	After freeze drying with trehalose (n = 3)	After free drying without trehalose (n = 3)
Size (nm)	46.8 ± 2.9	52.3	49.9	54.1 ± 2.0	53.9 ± 3.1	48.3 ± 3.6
Main peak (%)	99.6 ± 0.7	76.5	96.8	99.2 ± 0.3	98.6 ± 1.2	99.7 ± 0.5
PDI	0.089 ± 0.051	0.263	0.228	0.100 ± 0.020	0.157 ± 0.032	0.122 ± 0.042
Zeta potential (mV)	−12.1 ± 0.9	ND	ND	−5.8 ± 3.5	−1.1 ± 1.1	−4.7 ± 0.5

experiments.

3.4. Characterization of Ω-formulation

3.4.1. Stability study

A stability study analyzing sizes and polydispersity index of loaded etoposide and blank Ω-LNCs was performed over a period of 15 days at 4 °C temperature storage. The results of this study are presented in Fig. 2.

No change in size was observed over a period of 15 days for blank nanocapsules or etoposide loaded nanocapsules. The polydispersity index also remained lower than 0.1.

3.4.2. Hemolytic complement activation

Hemolytic complement activation is an *in vitro* test leading to an *in vivo* projection of interactions between the LNCs and the human plasma. The study was performed on the Ω-type LNCs loaded and non-loaded with etoposide. The results obtained (Fig. 3) were compared with two other formulations which are the PMMA nanospheres and the LNCs made with Labrafac® CC (Vonarbourg et al., 2006) known to be very activating and not activating, respectively.

Thanks to this result, it was possible to observe that the LNCs loaded with etoposide were not more complement activating than the non-loaded LNCs and they were both only slightly more activating than the LNCs made of Labrafac® CC.

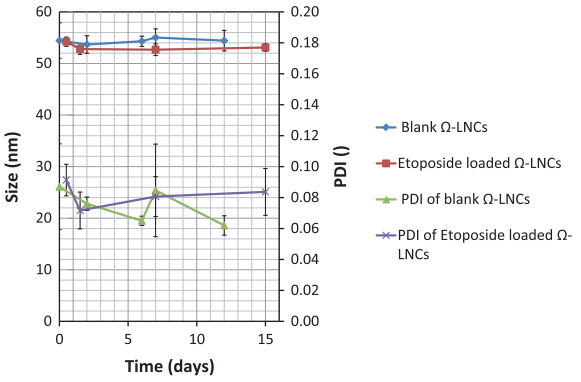

Fig. 2. Size and polydispersity index (PDI) of lipid nanocapsules (n = 3 batches for each formulation) loaded or non-loaded with etoposide along 15 days.

3.4.3. Release study

The release of etoposide through a membrane of 50 kD in a biomimetic environment was studied on three beakers for 72 h. The media contained protein at a concentration close to that of plasma. The amount of Ω-LNCs loaded with etoposide per unit volume was calculated to mimic the injection of a therapeutic dose (150 mg/m^2). Thus, the release of etoposide was found closer to *in vivo* conditions in the plasma. The HPLC results of the continuous medium are presented in Fig. 4.

3.5. In vitro cell viability

The CyQUANT® Cell Proliferation assay was used to determine *in vitro* cytotoxicity after exposure of Ω-LNCs (with or without etoposide) to both cells lines H209 and H69AR with a cascade concentration range of etoposide (0.01–100 μM). A short exposure time (72 h) was deliberately chosen to only observe the cytotoxicity activity of the LNC on cells (no impact of culture conditions on cell viability). The cell survival profiles of the H209 cells and H69AR cells exposed on etoposide-LNCs, blank-LNCs and free etoposide were illustrated in Figs. 5 and 6, respectively.

H209 cell culture studies revealed that at low concentrations (0.01–0.1 μM), neither etoposide solution nor LNCs (blank or etoposide loaded) altered the cell growth. At 1 μM concentration, the cell viability was decreased by the addition of etoposide loaded LNC and etoposide solution, while blank nanocapsules had no effect on it. At higher concentrations (10–100 μM), cell survival was dramatically decreased for blank or etoposide loaded LNCs,

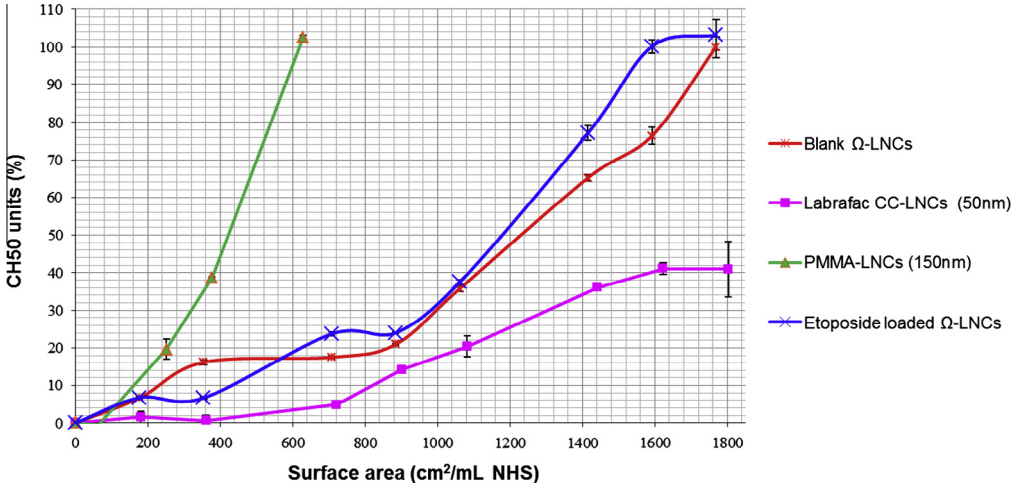

Fig. 3. Complement activation in function of specific surface area of different nanocarriers: blank or etoposide loaded lipid nanocapsules (Blank Ω-LNCs or etoposide loaded Ω-LNCs) see characteristics in text; Labrafac® CC nanocapsules (Labrafac® CC-LNCs) with a mean size of 50 nm; or PMMA nanoparticles (PMMA-LNCs).

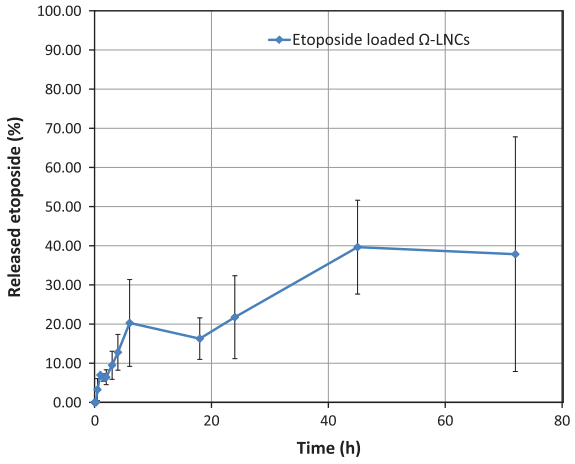

Fig. 4. Release study of etoposide from lipid nanocapsules (Ω-LNCs) over time in a medium mimicing plasma, $n = 3$.

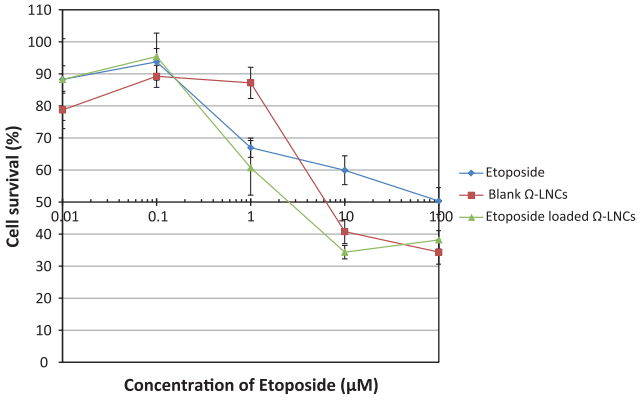

Fig. 5. Cyquant® cell survival assay on H209 cells after 72 h, relatively to the concentration of etoposide encapsulated or not in lipid nanocapsules (Ω-LNCs), $n = 3$.

resulting in an IC_{50} of about 2.5 μM and 6 μM, respectively. Moreover, the cytostatic effect of etoposide solution was lower since its IC50 was 100 μM.

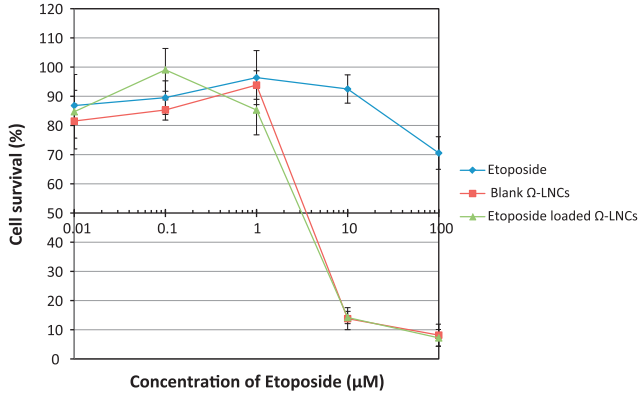

Fig. 6. Cyquant® cell survival assay on H69AR cells after 72 h, relatively to the concentration of etoposide encapsulated or not in lipid nanocapsules (Ω-LNCs), $n = 3$.

Table 6
Characteristics of LNCs obtained with the original Ω-formulation (see text for details) or after a 15× scale-up.

Quantity of materials	Ω-Formulation	
	Initial formulation ($n = 3$)	15× Scale-up ($n = 1$)
Size (nm)	54.1 ± 2.0	50.42
Main peak (%)	99.9 ± 0.2	100
Polydispersity index	0.100 ± 0.020	0.068
Zeta potential (mV)	−5.8 ± 3.5	−0.032
Encapsulation efficiency (%)	75.8 ± 3.3	80.1
Drug loading (mg/g)	5.7 ± 0.3	6.0

H69AR cell culture studies confirmed the resistant character of these cells at etoposide solution since a slightly cell survival decrease (over 70%) was observed at higher concentration ($IC_{50} > 100$ μM). On the other hand, blank-LNCs and etoposide-LNCs had similar toxicity on H69AR cells. Cell survival was dramatically decreased (below 20%) at the concentration of 10 μM, resulting in an IC_{50} of about 3 μM for both blank-LNCs and etoposide-LNCs.

3.6. Scale-up

Finally, a scale-up of the Ω-formulation was performed. The weight of all compounds was multiply by 15 folds. These LNCs were compared to Ω-LNCs, formulated at initial-scale, in terms of their drug loading and morphological characteristics (Table 6). No difference was observed on LNCs characteristics. These results indicate that Ω-LNs can be produced on a larger scale.

4. Discussion

One of the major challenges in drug nanoencapsulation consists in obtaining the higher drug loading in a smallest nanocarrier. Polymeric nanoparticles and solid lipid nanoparticles (SLN), which have a broad range of medical applications, were used to encapsulate etoposide. The first ones were formulated with biodegradable polymers such as polylactic acid (PLA) (Gaucher et al., 2007), polylactide-co-glycolic acid (PLGA) (Snehalatha et al., 2008; Yadav et al., 2011), polycaprolactone (PCL) (Snehalatha et al., 2008) or poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBHHX) (Kilicay et al., 2011) by nanoprecipitation or solvent evaporation techniques. These nanoparticles, whose size is greater than 100 nm, showed drug loading upper than 2% (w/w) and encapsulation efficiency up to 80%. The SLN (Reddy et al., 2006) or nanostructured lipid carriers (Patlolla and Vobalaboina, 2008; Zhang et al., 2011) are characterized by an encapsulation efficiency close to 100% for etoposide and a drug loading upper than 2% but high temperature (>75 °C) was necessary to obtain nanocarriers. Recently, encapsulation of the anticancer drug etoposide was achieved inside PEGylated poly(propylene imine) dendrimer and it was found that 20–21 molecules of drug are solubilized per dendritic polymer (Sideratou et al., 2010). Encapsulation of etoposide was also performed in polymeric micelles (Mohanty et al., 2010) or fatty acids micelles (Varshosaz et al., 2012). However, all these formulations required organic solvents that can be harmful for the patients.

In response to this problem, PLGA nanoparticles of etoposide were developed using fully biocompatible nanoprecipitation technique (Callewaert et al., 2012). For that, the polymer was dissolved in glycofurol, an injectable solvent. Unfortunately, encapsulation efficiency was dramatically decreased (<15%), probably due to the low extraction of the glycofurol by the external phase. Lipid nanospheres were also prepared by dissolving etoposide in oily components without any organic solvents (Patlolla and Vobalaboina, 2008) resulting in a lower drug loading generally observed for this encapsulation technique.

In a previous study performed by our group, etoposide encapsulation was obtained in LNCs, with Labrafac® CC as oily core, resulting in drug loading close to 2.7 mg/g dry nanoparticles and encapsulation efficiency up to 90% (Lamprecht and Benoit, 2006). However, etoposide was dissolved in this neutral oil prior to all the preparation steps, in presence of methanol. Despite those drawbacks, the obtained nanocapsules were very promising because they suppressed glioma cell growth *in vitro* and displayed P-glycoprotein inhibition. This latter interesting property has since been obtained on other cell lines with LNCs (Roger et al., 2010).

In view of the previous cited studies, there was thus still a need for a biocompatible etoposide nanocarrier, displaying a drug loading that allows therapeutic use of the drug. The aim of this study was to achieve etoposide loaded LNCs without any organic solvents and a higher drug loading. Results of solubility study showed that among oily excipients evaluated, Transcutol® HP was the best candidate to solubilize etoposide. So, an LNC formulation previously developed for Sn38 encapsulation (Roger et al., 2011) has been adapted, in order to obtain etoposide-LNCs formulation with an average size of 50 nm and a polydispersity index lower than 0.2.

Upon further optimization with drug loading and polydispersity as main targets, an etoposide-LNCs formulation, called Ω-LNCs, was obtained, which oily core was made of 20% Transcutol® HP, 60% Labrafil®M1944CS and 20% Labrafac® CC. These new LNCs display an average size of 54.1 ± 0.2 nm and a low polydispersity index of 0.10 ± 0.02. The obtained encapsulation efficiency was approximately 76% for a drug loading of 6 mg/g of dry LNCs (etoposide concentration was approximately 12 mg/g of lipid). Moreover, the present formulation differs from that developed for Sn38 on two main points. Firstly, the addition of Transcutol® HP containing dissolved etoposide, was not performed with all other components before the three heating–cooling cycles, but at the beginning of the last cooling, as described in one of our recent patent (Benoit et al., 2010). The second important improvement on this formulation is that, Lipoïd® S75-3 has been removed of original formulation to obtain what was called the Ω-LNCs formulation. This surfactant, a lecithin, was added initially to the LNCs formulation in order to reinforce the nanocapsule shell but it was difficult to solubilize and could generate an increase of polydispersity index. The stability study of the Ω-LNCs showed that the absence of Lipoïd® S75-3 in the formulation did not alter the LNC characteristics. Moreover, classical LNCs, i.e., with Lipoïd® S75-3, required addition of trehalose as cryoprotectant in order to preserve their monodisperse characteristics (Dulieu and Bazile, 2005). It has been possible to lyophilize the Ω-LNCs, in absence of cryoprotectant, without any change of size and polydispersity index after resuspension. In a precedent study, Vrignaud et al. (2011) observed the ability of their LNCs to bear a freeze-drying process without cryoprotectant, and without important change in their sizes and surface potentials. As Ω-LNCs studied here, these LNCs have been formulated in absence of Lipoïd® S75-3. Moreover, works about freeze-drying of nanoparticles mentioned that PEG could act as stabilizers, and protect the product during freeze-drying against the freezing and the drying stresses (Abdelwahed et al., 2006). Even if Lipoïd® S75-3 is anchored in the oily phase, the shell of the classical LNCs was made from a mixture of Solutol® HS15 and Lipoïd® S75-3. In the LNCs synthesized without Lipoïd® S75-3, the shell is only made of Solutol® HS15 and the PEG chains of this product probably act as cryoprotectant during the freeze-drying process.

After 3 days, the percentage of etoposide released was about 40% and reached a plateau. The characteristics of the release of etoposide obtained in this study were relatively close to those obtained by Lamprecht and Benoit (2006) with the same drug encapsulated in classical LNCs, i.e., with an oily core only composed of Labrafac® CC. On the other hand, there were very different from those obtained for camptothecin with a formulation of LNCs that was close to the Ω formulation (Roger et al., 2011). Thus, the first obtained a release of etoposide up to 60% after 24 h in a sink media buffered at 7.4 while the second described a release of about 7% over the same period in a phosphate buffered medium at the same pH and protein-free in non-sink conditions. These differences can be explained by the solubility profile of Sn38 and etoposide, $\log P = 2.65$ and 0.60 respectively and the presence of protein that helps dissolution process in the first study. The fact that our results, obtained in a media close to the one used by Lamprecht and Benoit (2006), are similar to what was obtained by those authors, underline that the drug release process was not affected by our new formulation.

The hemolytic complement activation provided predictive indicators for *in vivo* long-circulating behavior, according to the stealth properties of the nanoparticles. Ω-LNCs loaded or non-loaded with etoposide showed similar activating properties of the complement suggesting a real encapsulation of the drug. Moreover, a low activation of complement proteins was observed at low surface values (until 1000 cm^2), similar to that observed for standard 50-nm LNCs, i.e., LNCs whose oily core was only composed of Labrafac®

CC. In this area, corresponding to less than 20% of activation, it is recognized that the circulation time in plasma of LNCs would be acceptable for therapeutic treatment. However, this result has to be confirmed with *in vivo* evaluation, and it will be necessary to test the activity of LNCs loaded with etoposide on animals bearing a previously described tumor-type small cell lung cancer (Iochmann et al., 2012) to determine whether these objects are actually suitable for treatment.

Finally, the results of *in vitro* cells viability on non-resistant cells H209 showed that the etoposide encapsulation in Ω-LNCs formulation did not compromise its cytostatic effect. At 1 μM etoposide concentration, the encapsulated form even led to a slight decrease of the cells viability compared to the etoposide solution, while the blank Ω-LNCs remained without any cytotoxic effect. No effect of etoposide loaded LNCs was observed on H69AR resistant cells. In fact, H69AR cells were obtained after selection in culture with increasing concentration of adriamycin (Mirski et al., 1987). These cells expressed a multidrug resistant phenotype and do not over express p-glycoprotein as many other multiresistant cell lines. Our nanoparticles have been showed to inhibit p-glycoprotein (Garcion et al., 2006), this is another evidence that the resistance of H69AR cells is not linked to p-glycoprotein. The resistance is due to a decrease of susceptibility to drug-induced DNA damage (Cole et al., 1991), this is why the encapsulation in nanocapsules failed to reverse cell resistance to etoposide. Only intrinsic toxicity of blank LNCs was observed for the high concentrations evaluated (10–100 μM). Nevertheless, only *in vivo* experiments could correctly describe therapeutic benefit of Ω-LNCs.

5. Conclusions

This study demonstrated the feasibility of etoposide-LNCs and improvements over the original promising formulation developed in our group several years ago.

The new LNCs were formulated without any use of solvents compared to other systems encapsulating etoposide. The LNCs are adapted to the effect of tissue permeability and retention (EPR) because their diameter was close to 50 nm. Finally, even if they have a drug loading less than other systems, they have one of the most important encapsulation efficiency of etoposide ever reached. Adapted to scale-up and freeze drying, containing only GRAS excipients, these new formulations are suitable for industrial process.

The performances of this formulation are promising, and make these LNCs a good nanomedicine candidate for preclinical animal studies in the view to elaborate a new therapeutic treatment of SCLC.

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