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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