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Research paper

Lipid nanocapsules: Ready-to-use nanovectors for the aerosol delivery of paclitaxel

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ABSTRACT

Aerosol drug delivery permits the development of dose-intensification strategies in severe, malignant lung diseases. The aim of the study was to demonstrate that the encapsulation of paclitaxel in lipid nanocapsules (LNCs), a novel drug nanocarrier for lipophilic components, allows one to provide pulmonary drug delivery of paclitaxel by nebulisation, thereby allowing preclinical and clinical studies. LNC dispersions are made into aerosols with commercial nebulisers. The structure, drug payload and cytotoxicity of nebulised LNCs were compared to fresh LNCs. The results demonstrated that LNC dispersions could be made into aerosols by using mesh nebulisers without altering the LNC structure. Only eFlow® rapid-produced aerosols are compatible with human use: the mean duration to nebulise 3 ml of LNC dispersion is less than 9 min, with an aerosol mass median aerodynamic diameter equal to 2.7 ± 0.1 μm and a fine-particle fraction (between 1.0 and 5.0 μm) of 81.5 ± 3.1%. No modifications of drug payload or cytotoxicity effects of paclitaxel-loaded LNC (PTX–LNC) were observed. In order to carry out preclinical studies, a scaled-up LNC formulation protocol was used. Chemical parameters, such as acidity and osmolarity, were optimised, and a storage procedure for PTX–LNC batches was set-up. Animal studies are now needed to determine the tolerance and therapeutic potential of LNC dispersion aerosols.

1. Introduction

Aerosol drug delivery constitutes an emerging field of knowledge with considerable interest for human medicine [1]. Whereas this method of administration is mandatory for asthma, the incorporation of a drug or a drug carrier into breathable droplets becomes more and more interesting to treat other bronchial and pulmonary diseases such as lung tumours [2]. Preclinical studies in thoracic oncology have demonstrated that this route of administration allows the delivery of a large quantity of chemotherapy agents directly into the lower airways and lungs of a patient with minimal systemic diffusion of the drug [3]. This dose-intensification strategy allows the promotion of an anti-tumoural effect with a reduced quantity of drug and with reduced or no systemic side effects. Some clinical studies of aerosol chemotherapies have been published [2]. A few clinical studies are ongoing and are registered on the ClinicalTrial.gov website [4].

Nevertheless, the design and the production of a new treatment to be administered by aerosol for human use is a complex process. First, the therapeutic properties of the drug (and the drug carrier) must be unchanged following the nebulisation process. Secondly, the aerosol must be mainly composed of 1–5 μm droplets in aerodynamic diameter, and the duration to nebulise the targeted dose must be less than 15 min. Thirdly, the nebulised solution must conform to precise pH and osmolarity characteristics. Some products, such as hypoosmolar or acid solutions, that induce coughing and bronchospasms are poorly tolerated [5–8]. Finally, the solution must be produced in large quantities and must be stable during storage for several weeks in order to allow preclinical and clinical studies to take place. All these points must be addressed to produce a nebulisable drug solution or drug carrier solution compatible with human use and with marketable insurance quality standards.

Lipid nanocapsules (LNCs) are patented nanocarriers designed to encapsulate lipophilic drugs without organic solvents. Their synthesis is based on an original phase-inversion process, allowing the production of nanocarriers in a sodium hydroxide aqueous solution with a size ranging from 25 to 100 nm [9]. The size distribution of

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the carrier is unimodal with a low polydispersity index. The LNC structure is composed of a lipid core in which the solubilised lipophilic drug is limited by a membrane of lecithin and pegylated poly-ethyleneglycol hydroxystearate (PEG) chains conferring some degree of stealthiness [10]. All the excipients of the LNC are FDA approved. At a temperature of 37 °C, the core is liquid, whereas the membrane is rigid. Previous studies have demonstrated the ability to entrap amiodarone [11], ibuprofen [12], tripentone [13], etoposide [14], docetaxel [15] and paclitaxel [16,17] into LNCs. In preclinical studies, etoposide and paclitaxel-loaded LNC showed a higher cytotoxicity effect than free drugs after systemic administration. This can be explained by sustained drug release and P-glycoprotein (P-gp) inhibition [14–16]. Interestingly, radioactive components such as ^{99m}Tc and ^{188}Re can be used to label LNC allowing the imaging of the distribution for diagnostic and therapeutic uses [18].

Taxol®, the first worldwide available formulation of paclitaxel, was a major step in therapeutic progress in the systemic treatment of ovarian, breast and non-small cell lung cancer patients. Paclitaxel, its active component, has many pharmacological effects that are particularly well adapted to cancer treatment. Paclitaxel is an antiproliferative [19], antiangiogenic [19], antimetastatic [20] and proapoptotic drug [21]. Nevertheless, paclitaxel is also a highly hydrophobic drug; in order to be solubilised; it requires the addition of dehydrated ethanol and Cremophor®EL (polyoxyl 35 castor oil) to allow it to be solubilised in water. This last excipient can induce shock syndrome after intravenous injection in humans due to histamine release in spite of medical preparation, and is not suitable for aerosol administration because of its high viscosity. For these reasons, Cremophor®EL-free formulations of paclitaxel are expected to show less toxicity for patients during chemotherapy treatment, improving patient tolerance [22] and aerosol delivery strategies. Over the last two decades, paclitaxel has been formulated into liposomes [23]. Systemic delivery has been well tolerated in mice and significant tumour growth inhibition has been observed in a subcutaneous murine model [24]. Aerosols of liposomal paclitaxel formulations have been developed and were effective on tumoural models of animal lungs and were found to significantly reduce tumoural growth [25,26]. These studies proved the high potential of aerosol paclitaxel for its administration in lung tumours and have stimulated further studies.

The aim of the present study was to demonstrate that the encapsulation of paclitaxel in LNC allows the pulmonary drug delivery of paclitaxel by nebulisation in order to carry out preclinical and clinical studies. First, the production of LNC dispersions was scaled-up, and the determinant physical and chemical parameters were optimised for aerosol delivery. Secondly, the storage of paclitaxel-loaded LNC dispersions was set-up. Finally, the feasibility of LNC aerosol of dispersions was studied.

2. Methods and materials

2.1. Materials

Captex® 8000 (glyceryl tricaprylate), Lipoïd® S75-3 (soybean lecithin at 69% of phosphatidylcholine) and Solutol® HS15 (polyglycol ester of 12-hydroxystearic acid and polyethylene glycol) were purchased from Abitec Corp (Colombus, OH, USA), Lipoid GmbH (Ludwigshafen, Germany) and BASF (Ludwigshafen, Germany), respectively. Paclitaxel powder was provided by AMPAC Fine Chemicals (Rancho Cordova, CA, USA). Distilled water from Cooper (Melun, France) was used. Sodium chloride (NaCl) was obtained from Prolabo VWR International (Fontenay-sous-bois, France). Taxol® 6 mg/ml solutions were obtained from Bristol-Myers Squibb (Rueil-Malmaison, France). Methanol and tetrahydrofurane were of high-performance liquid chromatography (HPLC) grade and were from Fischer Scientific (Elancourt, France) and Carlo Erba Reactifs (Val de Reuil, France), respectively. Ministar 0.20 μm high-flow filters from Sartorius AG (Goettingen, Germany) were used. Cryotubes CRYO.S were purchased from Greiner Bio.one (Frickenhausen, Germany).

2.2. LNC formulation

The study was performed on 55-nm-diameter LNC prepared according to an original method already described elsewhere [9]. Briefly, Captex® 8000, Lipoïd® S75-3, Solutol® HS 15, NaCl and water (1.2 g; 67.2 mg; 1.0 g; 73.3 mg and 1.8 g, respectively) were mixed and heated under magnetic stirring to 90 °C. Three cycles of heating and cooling between 90 °C and 70 °C were carried out followed by fast cooling induced at 78 °C by adding 13.2 ml of 0 °C deionised water. Magnetic stirring was applied to the suspension of LNC for 5 min at room temperature.

For the formulations of paclitaxel-loaded LNC, 1.8% of paclitaxel powder was first solubilised in Captex® 8000 (i.e. 21.6 mg in 1.2 g) under magnetic stirring and heated at 50 °C for 30 min. This rate is inferior to the solubility threshold of 2.0% of paclitaxel in Captex® 8000 (personal data). The same method was then applied to this mixture.

2.3. Size distribution

The LNCs were analysed for size distribution by photon-correlation spectroscopy and zeta potential using Malvern Zetasizer®, Nano Series DTS 1060 (Malvern Instruments S.A., Worcestershire, UK) after filtration through a 0.20-μm Sartorius filter.

2.4. Drug payload and encapsulation efficiency

Three samples of each batch of paclitaxel-loaded LNCs were filtered by 0.20-μm Ministar high-flow filters in order to remove insoluble paclitaxel crystals. Then, paclitaxel-loaded LNC samples were prepared by dissolving a determined quantity of LNC dispersion in a 96/4 (v/v) methanol/tetrahydrofurane solution. Aliquots of 50 μl and 500 μl were used for the payload study and for the aerosol characterisation, respectively. High-performance liquid chromatography (HPLC) was performed using a Waters Alliance® 2690 system (Waters S.A., Saint-Quentin en Yvelines, France). A 20-μm aliquot of each filtrate was injected in triplicate into an HPLC XTerra® RP18 column 5 μm 4.6 mm × 150 mm (Waters S.A.). The mobile phase consisted of water and acetonitrile. Paclitaxel was eluted using a gradient elution programme of 50–80% acetonitrile. The flow rate was 1.0 ml/min. Data were analysed by Empower Pro software, version 5.00. Good linearity was observed in the range from 39.0 to 390.2 mg/l with a correlation coefficient above 0.99. The detection limit was 10.0 mg/ml and the quantification limit was 20.0 mg/ml. The paclitaxel peak was observed at 2 min 42 s. A diode array detector was used to identify the paclitaxel peak.

2.5. Scaled-up procedure

A scaled-up procedure was used by proportionately increasing the quantities of all the compounds. Thermal exchanges during the heating and cooling steps were optimised by immersing glasses in water at 95 °C and room temperature. The rest of the procedure remained unchanged.

2.6. Osmolarity studies

Osmolality was measured using a 5520 Vapro vapour osmometer from Wescor (Logan, Utah, USA).

2.7. Acidity studies

Using calibrated micro-pH CRISON 2001 OSI (Paris, France), pH measurements were taken.

2.8. Sterilisation procedure

Each formulation was filtered through a 0.20-μm Sartorius filter into a laminar flux hood and aliquoted in 2 ml sterile and apyrogene cryotubes CRYO.S before storage.

2.9. Sterility and apyrogenicity controls

Aliquots of 100 μl of blank LNC dispersions were plated onto trypticase soy agar with 5% horse blood. After overnight incubation at 37 °C, bacterial colonies were counted and their number was expressed as colony-forming units (CFU)/ml. Dispersions of fresh blank LNC dispersions were stored at +4 °C, and the tests were performed once a month for 4 months. Lipopolysaccharide (LPS) levels in fresh blank LNC dispersions were quantified by using Limulus Amebocyte Lysate from Endosafe Charles River (Charleston, USA), according to the manufacturer's protocol [27]. Results were negative when the LPS level was below 0.25 UI/ml.

2.10. Storage protocol

The storage protocol was based on a freezing method. The cryotubes CRYO.S, previously filled with LNC dispersions, were frozen in liquid nitrogen. The thawing protocol consisted of thawing the cryotubes CRYO.S at room temperature for 30 min. After the protocol was completed, the size distribution, dispersal, potential zeta, pH, osmolarity, drug payload and cytotoxicity of the LNC dispersions were checked.

2.11. Nebulisation tests

Our aim was to determine how to produce an aerosol of LNC dispersions compatible with human use.

A two-step procedure was used. First, the nebulisation of blank LNCs was carried out to determine which nebulisers were able to produce an aerosol without significant modification of the size and polydispersity of the LNC dispersions in less than 15 min. Six different nebulisers were tested with their specific power supply: two jet nebulisers: atomisor NL9M nebuliser (Diffusion Technique Française, France) and PARI LC sprint nebuliser (PARI, Germany); two ultrasonic nebulisers: Atomisor Megahertz nebuliser (Diffusion Technique Française, France) and Multisonic® nebuliser (Otto Schill, Germany); and two mesh nebulisers: Aeroneb® Pro nebuliser (Aerogen, CA, USA) and eFlow® rapid (PARI, Germany). For each apparatus, 3 ml of the blank LNC dispersions was loaded (in triplicate) before gathering samples in the liquid phase (1.0 ml of distilled water) with an impinger (Ace Glass Inc., Vineland, USA) operating at an airflow rate of 12.0 l/min. The duration of the nebulisation was timed. The size and polydispersity of nebulised LNC collected into the impinger at the end of the process were evaluated by photon-correlation spectroscopy using Malvern Zetasizer®, Nano Serie DTS 1060 (Malvern Instruments S.A., Worcestershire, UK).

For the second-step of evaluation, only the devices selected from the first step were tested to study the drug payload and cytotoxicity of the paclitaxel-loaded LNC dispersions after nebulisation, to determine the aerosol mass median aerodynamic diameter (MMAD) and the fine-particle fraction (FPF). Paclitaxel-loaded LNC dispersions were nebulised (in triplicate) to gather samples in a liquid phase with an impinger (Ace Glass Inc., Vineland, USA) operating at an airflow rate of 12.0 l/min. Volumes of nebulised samples and residual volumes were measured. The aerosol output rate was calculated. Samples of nebulised paclitaxel-loaded LNC dispersions and of paclitaxel-loaded LNC dispersions remnants were sampled to assay the drug payload by using the previously mentioned HPLC protocol and to carry out cytotoxicity assays. A Next Generation Pharmaceutical Impactor model 170 cascade impactor (MSP Corporation, Shoreview, MN, USA) was used to measure the size distribution of droplets at a flow rate of 15.0 l/min. The deposited aerosol on each impactor stage was re-suspended in 5 ml of distilled water. The drug payload of aerosol samples was quantified by the HPLC protocol. The MMAD and FPF levels between 1.0 and 5.0 μm were determined using cascade impaction data according to European Norm (EN) 13544-1;2007 from three independent experiments.

2.12. In vitro cytotoxicity study

The *in vitro* cytotoxic activity levels of fresh and thawed blank LNC, fresh and thawed paclitaxel-loaded LNC, and nebulised paclitaxel-loaded LNC against NCI-H460 human lung cancer cells were determined using a growth inhibition assay and compared with Taxol® and fresh non-nebulised paclitaxel-loaded LNC. Taxol® was supplied in vials containing 6 mg of solution for injection. The tested solution was obtained by diluting Taxol® in 0.9% NaCl for a final concentration of 1.2 mg/ml. To check the cytotoxic effect of the nebulised paclitaxel-loaded LNC dispersions, we compared two methods. First, we used the method previously described by Gagnadoux et al. on gemcitabine [28]. According to this method, samples were taken from the nebuliser reservoir before (T0) and after various nebulisation times (2, 4, 6 min and the end of nebulisation). Secondly, these tests were carried out with nebulised LNC dispersions sampled in an impinger at the end of complete nebulisation. A NCI-H460 human, large-cell lung carcinoma cell line was obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were cultured in RPMI 1640 medium containing glutamine (Lonza, Verviers, Belgium), 10 mM HEPES (Sigma Chemical Co., Saint Louis, USA), 1 mM sodium pyruvate (Lonza, Verviers, Belgium), 1.5 g/L bicarbonate (Cambrex Bio Sciences, Verviers, Belgium), 10% fetal bovine serum (Lonza, Verviers, Belgium), 50 U/ml penicillin, and 50 mg/ml streptomycin (Sigma–Aldrich Co., Ayrshire, UK). Cells were routinely maintained at 37 °C in a humidified atmosphere of 5% CO_2 in air. The culture medium was replaced every 2 or 3 days, and cells were subcultured weekly using 0.25% trypsin – 1 mM EDTA (Sigma–Aldrich Co., Ayrshire, UK). Tumour cells were seeded in 24-well plates at 50,000 cells/well. All assays were performed with exponentially growing cultures. After 48 h, a drug-containing medium was added. For each sample, with a dose range from 10^{-3} to 100 nM of paclitaxel encapsulated LNC or of blank LNC formulations was tested. The cytotoxicity was assessed after 2 days of exposure by adding 80 μl of CellTiter 96® Aq_{ueous} One Solution Reagent (Promega, Madison, WI) to each well according to the manufacturer's instructions [29,30]. After 4 h in culture, the cell viability was determined by measuring the absorbance at 492 nm using a Multiskan Ascent microplate reader (Thermo Fisher Scientific Cergy-Pontoise, France). Because LNC dispersions are not translucent, the absorbance of wells containing the cells was subtracted from the absorbance of wells containing culture subtract alone for each point. The concentration of the drug causing 50% growth inhibition (IC50) was calculated. Three independent experiments were performed in triplicate.

2.13. Statistical analysis

The results are expressed as a mean ± SD. For the analysis of statistical significance, the Kruskal–Wallis test was applied with Bonferroni's correction. In all cases, $P < 0.05$ was considered to be significant.

3. Results

3.1. LNC formulations with a scaled-up procedure

The size distribution of LNC dispersions obtained by a scaled-up procedure was compared to the standard protocol. For all the scaled-up batches, the size distribution was unimodal. The mean value and standard deviation of particle size, polydispersity index and zeta potential of triplicate measurements were unchanged for both blank and paclitaxel-loaded LNC dispersions ($P > 0.05$). Results of blank LNC dispersions are presented in Table 1. Results of paclitaxel-loaded LNC are presented in Table 2.

3.2. Sterility and pyrogenicity tests

Three different scaled-up formulations of blank LNC dispersions were sterile for up to 4 months. No additional sterility control was performed beyond this time point. These sterile formulations were non-pyrogenic.

3.3. Osmolarity and pH studies

The pH and osmolarity levels were measured in triplicate in fresh blank LNC formulations at 20 °C. The mean pH values were 5.93 ± 0.05 and 5.92 ± 0.04 with standard and scaled-up protocols, respectively. The mean osmolarity values were 253 ± 2 mOsm/kg and 253 ± 3 mOsm/kg with standard and scaled-up protocols, respectively. Results were similar for paclitaxel-loaded LNC produced by the standard and the scaled-up protocol (data not shown). Because these two chemical parameters can be aggressive to bronchial and alveolar cells and can induce adverse effects, the pH of the LNC dispersion was increased to reach the tissue's pH by adding molar sodium hydroxide solution; the osmolarity was also increased to a near-human iso-osmolarity value by adding sodium chloride. These adjustments were made after LNC fabrication. By adding 10 μl of molar sodium hydroxide solution per 30 ml of final volume LNC dispersion and 15% of sodium chloride (calculated on the formulation protocol), a pH value of 7.28 ± 0.02 and an osmolarity level of 267 ± 2 mOsm/kg were obtained. The pH and the osmolarity levels of all the LNC dispersions were modified as described above for further experiments.

3.4. Storage procedure

The mean particle size, polydispersity index and zeta potential of blank and paclitaxel-loaded LNC dispersions were measured before and after freezing in liquid nitrogen for several hours. The visual aspect of all the thawed LNC dispersions was identical to fresh LNC dispersions. Results are presented in Table 3. The osmolarity and pH of blank and paclitaxel-loaded LNC dispersions were unchanged after freezing in liquid nitrogen (data not shown).

3.5. Aerosol tests

For first step studies, a thawed blank LNC batch of 65.7 nm with a 0.05 polydispersity index was tested with Atomisor NL9M, Atomisor Megahertz and eFlow® rapid nebulisers. No cloud was seen with the Atomisor Megahertz device. The results are presented in Table 4.

To complete these results, a different thawed, blank LNC preclinical batch of 84.2 nm with a 0.14 polydispersity index was tested with PARI LC Sprint, Multisonic® and Aeroneb® Pro nebulisers. No cloud was seen with the Multisonic® device. The results are presented in Table 5.

We considered that the results of step one proved that jet nebulisers greatly disorganise LNC dispersion which lose their unimodal size distribution. Likewise, we observed that ultrasonic devices were unable to produce an aerosol with LNC dispersions. For these reasons, neither jet nor ultrasonic nebulisers were selected for further studies. Two different mesh nebulisers were able to produce aerosols of LNC dispersions without breaking the LNC. The duration of nebulisation was 8.4 and 62.3 min. for eFlow® rapid and Aeroneb® Pro, respectively. For this reason, Aeroneb® Pro was not selected for the second-step analysis.

The second-step analysis was performed with eFlow® rapid only. The residual volume in the medication reservoir was 1.08 ± 0.06 ml. The nebulised volume (including 1 ml of distilled water) was 2.13 ± 0.06 ml. The aerosol output rate was 0.22 ± 0.01 ml/min. The MMAD was 2.7 ± 0.1 μm. The FPF between 1.0 and 5.0 μm was 81.5 ± 3.1%.

3.6. Drug payload

Typical results of HPLC are presented in Fig. 1. A paclitaxel peak can be observed at 2.5 min (shown by an arrow). Paclitaxel payload in fresh paclitaxel-loaded LNC was equal to 1.65 mg/ml ± 3.0%. The encapsulation efficiency is 100% for all fresh paclitaxel-loaded LNC dispersions. Because eFlow® rapid has a residual volume in the medication reservoir, the payload was obtained by adding the quantity of paclitaxel measured to the residual volume collected in the reservoir after nebulisation and the quantity of paclitaxel measured in the nebulised volume collected in the impinger bottle. The paclitaxel payload in the residual volume was unchanged in comparison with the non-nebulised solution. The paclitaxel's concentration in the nebulised volume was equal to 1.45 mg/ml ± 1.9%. Quantification results were expressed as a percentage of drug payload in comparison with fresh paclitaxel-loaded LNC. The amount of paclitaxel was 100.0 ± 4.9%, 102.4 ± 3.0% and 98.2 ± 2.1% for fresh paclitaxel-loaded LNC thawed after freezing in liquid nitrogen, fresh paclitaxel-loaded LNC, and eFlow® rapid nebulised paclitaxel-loaded LNC, respectively.

3.7. Cytotoxicity tests

The results of cytotoxicity assays are presented in Figs. 2 and 3. Taxol® IC50 was 4.4 nM as published elsewhere (4.8 nM) [31]. For frozen Taxol® in liquid nitrogen, the cell survival curve and the IC50 were unchanged. The cytotoxic effects of fresh paclitaxel-loaded LNC dispersion were similar to Taxol® and the IC50 was 3.8 nM (Fig. 2). The cytotoxic effect was unchanged for paclitaxel-loaded LNC frozen in liquid nitrogen (Fig. 2). Interestingly, the IC50 of fresh blank LNC dispersion was 5.4 nM (Fig. 2).

Table 1
Characterisations of blank LNCs produced by the scaled-up procedure. Experiments performed in triplicate.

	Multiplier	Mean particle size (nm)	Polydispersity index	Zeta potential (mV)	Volume (ml)
Standard protocol	None	50.4 ± 0.7	0.052 ± 0.011	−4.85 ± 0.55	15
Scaled-up procedure #1	10	52.4 ± 0.5	0.053 ± 0.019	−5.76 ± 0.40	150
Scaled-up procedure #2	10	53.8 ± 1.2	0.041 ± 0.010	−5.64 ± 0.94	150
Scaled-up procedure #3	10	53.9 ± 1.2	0.055 ± 0.013	−4.54 ± 0.15	150
Kruskal–Wallis test	–	$P > 0.05$	$P > 0.05$	$P > 0.05$	–

Table 2
Characterisations of paclitaxel LNC produced by the scaled-up procedure. Experiments performed in triplicate.

	Multiplier	Mean particle size (nm)	Polydispersity index	Zeta potential (mV)	Volume (ml)
Standard protocol	None	49.0 ± 1.8	0.061 ± 0.017	−6.51 ± 0.25	15
Scaled-up procedure #4	4	53.4 ± 1.9	0.078 ± 0.023	−6.94 ± 0.56	60
Scaled-up procedure #5	10	48.4 ± 0.5	0.038 ± 0.010	−4.53 ± 0.15	150
Kruskal–Wallis test	–	$P > 0.05$	$P > 0.05$	$P > 0.05$	–

Table 3
Characterisations of blank and paclitaxel LNCs after the conservation protocol in liquid nitrogen.

	Mean particle size (nm)	Polydispersity index	Zeta potential (mV)
Fresh, blank LNCs	53.3 ± 0.5	0.06 ± 0.01	−5.12 ± 0.32
Thawed, blank LNCs	65.7 ± 1.8	0.05 ± 0.01	−5.66 ± 0.19
Fresh, paclitaxel-loaded LNC	53.4 ± 1.9	0.08 ± 0.02	−5.84 ± 0.87
Thawed, paclitaxel-loaded LNC	67.5 ± 0.5	0.07 ± 0.01	−5.26 ± 0.49

Table 4
Characterisations of a blank LNC of 65.7 nm with a 0.05 polydispersity index after nebulisation by three different devices.

Nebuliser	Mean particle size (nm)	Polydispersity index	Duration (min) mean ± SD
Atomisor NL9 M	Trimodal distribution of LNC	–	7.5 ± 2.4
Atomisor Megahertz	Signal too low because dispersion was too diluted	–	10.1 ± 0.2
eFlow® rapid	83.8	0.17	8.4 ± 0.4

Table 5
Characterisations of a blank LNC of 84.2 nm with a 0.14 polydispersity index after nebulisation by three other different devices.

Nebuliser	Mean particle size (nm)	Polydispersity index	Duration (min) mean ± SD
PARI LC Sprint	Trimodal distribution of LNC	–	5.5 ± 0.9
Multisonic®	Signal too low because dispersion was too diluted	–	11.3 ± 0.6
Aeroneb® Pro	84.9	0.20	62.3 ± 4.0

For nebulised paclitaxel-LNC, the growth inhibition curves were similar to fresh paclitaxel-loaded LNC dispersion (Fig. 3). The IC50 parameters were also identical.

4. Discussion

The commercial formulation Taxol® cannot be nebulised because of the presence of Cremophor®EL used as a solvent for paclitaxel. For this reason, promising drug intensification strategies by aerosol therapy in lung malignancies cannot be expected from Taxol® in its current commercial form. The present study demonstrates that a new formulation of paclitaxel without Cremophor®EL, in which paclitaxel is encapsulated in a nanovector, allows a suitable form of paclitaxel aerosol to be produced. This aerosol, composed of water droplets in which the LNCs are dispersed, is compatible with a therapeutic study in humans, and could permit the research of new therapeutic effects of paclitaxel in pulmonary delivery strategies for both primary and secondary lung malignancies.

A scaled-up LNC formulation protocol was used in order to produce LNC batches adapted to preclinical and clinical studies. The standard formulation protocol produced 15 ml of LNC dispersion per batch. This volume was insufficient to carry out repeated *in vivo* administrations of LNC. The scaling-up of the standard formulation protocol was obtained by increasing the proportions of all the components by a multiple of 10. The main modification was the optimisation of thermal exchange by water baths. The characterisation of the LNC produced by the scaled-up protocol by diffraction laser revealed no differences with the LNC produced with the standard protocol.

Osmolarity, acidity, sterility and pyrogenicity, the main chemical and biological parameters of the LNC dispersions particularly involved in tolerance of aerosols in humans, were studied. Previous published data have demonstrated that hypotonic aerosols may

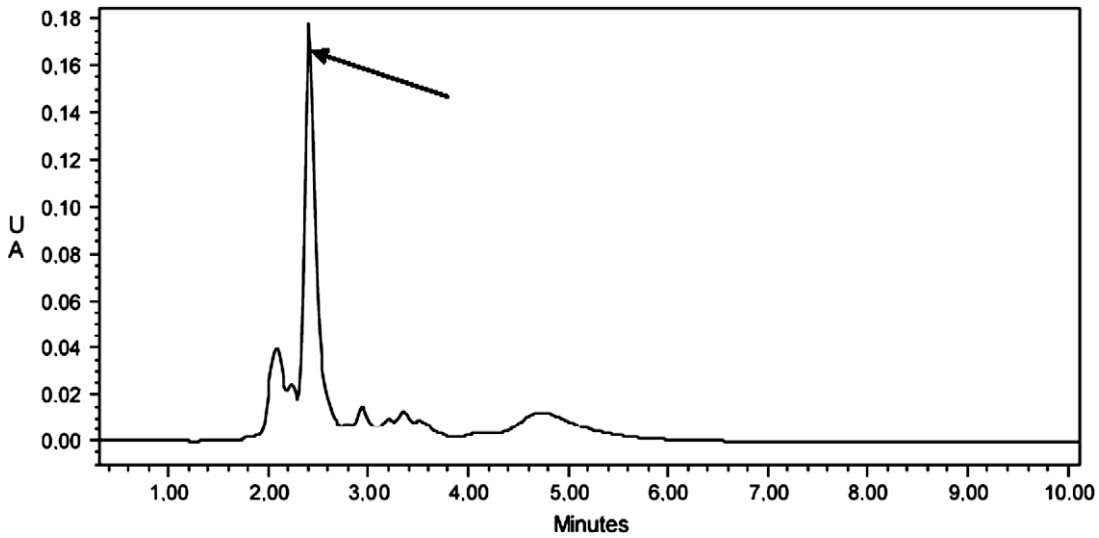

Fig. 1. A typical chromatogram of paclitaxel exhibiting a retention time of about 2.5 min (arrow).

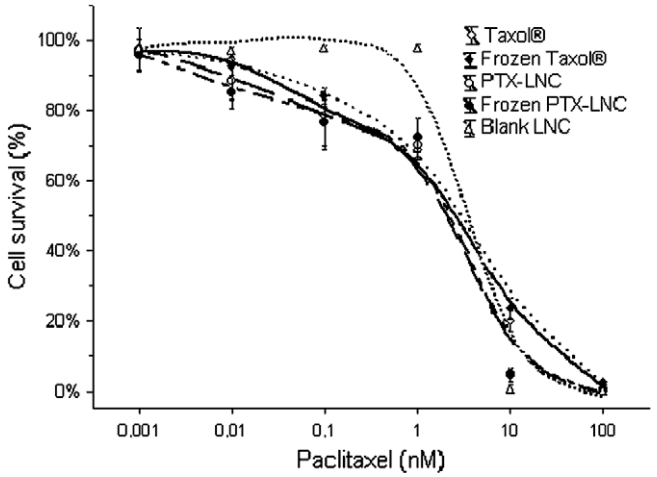

Fig. 2. Growth inhibition assay of Taxol®, Taxol® previously frozen in liquid nitrogen, PTX–LNC, PTX–LNC stored in liquid nitrogen and blank LNC in NCI-H460.

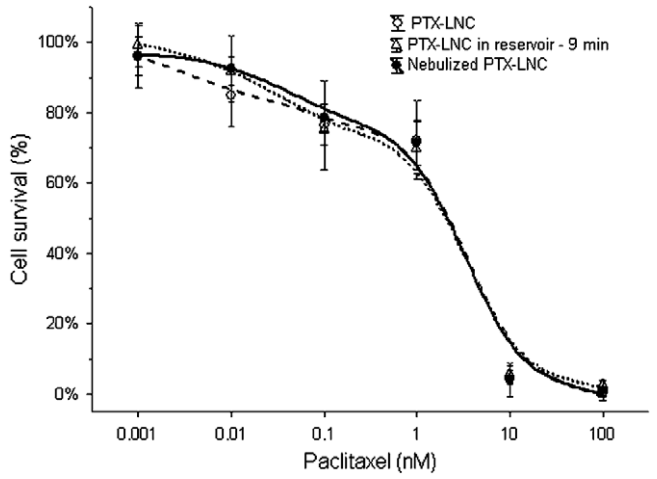

Fig. 3. A growth inhibition assay of non-nebulised and nebulised PTX–LNC on NCI-H460. Nebulised PTX–LNCs were sampled in the impinger at the end of the nebulisation process.

induce coughing [5,6] and that acidic aerosols (pH = 5) may trigger significant alteration of pulmonary functions due to bronchospasms [7]. Moreover, acidity and hypo-osmolarity could exacerbate these deleterious bronchial effects [8]. It was, therefore, considered necessary to assess these parameters for LNC dispersions. The osmolarity was not measured by the technique of 'freezing point depression', the reference method for solutes or ions in water, but by a vapour–pressure method. This method was chosen as it does not modify the physical status of the sample, a key point for colloid dispersion and LNC dispersion. Hypo-osmolarity of the LNC dispersions (253 mOsm/kg) was compensated by the adjunction of sodium chloride after the formulation until the level of 270 mOsm/kg was reached. This threshold was chosen to avoid the theoretical risk of flocculation. Acidity was compensated for by adding a small quantity of molar sodium hydroxide solution at the end of the formulation. This step did not modify the osmolarity value. LNC formulations were demonstrated to be sterile and apyrogenic with two conventional tests. These results showed that LNC dispersions could display the principal chemical and biological parameters required for aerosol delivery in humans.

A new PTX–LNC storage protocol utilising a freezing procedure in liquid nitrogen was described. A freeze-drying storage protocol of the LNC dispersion was not used because there was a risk of environmental contamination by paclitaxel during the sublimation step and a risk of LNC sterility loss during the rehydration step [32]. Because LNCs are slow-release carriers, it was mandatory to demonstrate that the storage protocol was able to suspend the drug release [16,33]. Small volumes of LNC and PTX–LNC dispersions were stored in liquid nitrogen for 4 months. After thawing, the zeta potential remained unchanged but the mean size increased by 15%, this supposedly being related to the increase of hydration of the PEG chains. The polydispersity index was multiplied by a factor of two. However, this index remained below 0.1, thus the size distribution of the LNC dispersions remained unimodal and log-normal. The drug payload of thawed PTX–LNC was found to remain unchanged. As the PTX–LNC dispersions were filtered before quantification with an HPLC protocol, the possible paclitaxel crystals remaining in the water phase, and thus non-encapsulated, were removed. This methodological point confirmed that the quantified paclitaxel was encapsulated and proved that the freeze-storage protocol had not destroyed the PTX–LNC and had effectively stopped the drug release process. After freezing storage, the cytotoxic effect of the PTX–LNC was identical to fresh PTX–LNC and the chemical parameters were not modified. These results demonstrated that the LNC storage protocol in liquid nitrogen did not alter the PTX–LNC and was well suited to conserve them.

The nebulisation studies were performed with commercial nebulisers on power input and showed that only mesh nebulisers were able to produce aerosols for LNC dispersion. Jet nebulisers seemed to disorganise the LNC size distribution which did not remain unimodal after the nebulisation process. This observation may reflect a phenomenon of destruction–rearrangement of LNC caused by the stress of the jet effect that has been applied to the LNCs several times before been aerosolised. Because LNCs encapsulate lipophilic drugs that precipitate in water, jet nebulisers cannot be used to nebulise LNC dispersions. The ultrasonic nebulisers were unable to nebulise a detectable quantity of LNCs. The two mesh nebulisers tested succeeded in producing LNC aerosols from LNCs dispersions. As these devices produce aerosols by a vibrating process without recirculation system, the LNCs have endured the nebulisation stress during the droplet formation only one time. This characteristic could explain the absence of disorganisation of the LNCs structure with mesh nebulisers contrary to jet nebulisers. The Aeroneb® Pro nebuliser did not modify the mean size distribution of the LNC but was not compatible with human use because the duration of nebulisation for 3 ml of LNC dispersion took over 1 h. A recent study has demonstrated that the output rate of Aeroneb® nebuliser was inversely correlated with viscosity of the nebulised solution [34]. As with all colloidal dispersion, the LNC dispersions had a higher viscosity than saline solutions; this could explain why the output rate was so low. On the contrary, the eFlow® rapid nebuliser was able to nebulise 3 ml of LNC dispersion in less than 9 min. No modifications of the drug amount and the cytotoxic effect on NCI-H460 cell line was observed even if the mean size of the LNC was increased by 25% and the polydispersity index was multiplied by three. The MMAD and the FPF rates (between 1.0 and 5.0 μm) were 2.7 μm and 81.5%, respectively. These main aerosol parameters were found to be highly compatible with the bronchial target for human use. The eFlow® rapid was considered as the only tested device able to nebulise LNC dispersions with good characteristics for human use.

Pulmonary drug delivery of paclitaxel by nebulisation is a new and highly potential therapeutic strategy [22]. Nebulisation of paclitaxel was first carried out from formulations of paclitaxel solubilised in organic solvents [26,35]. Recently, formulations of paclitaxel encapsulated in liposomes have been tested in preclinical studies. Koshkina et al. tested a liposomal formulation of

paclitaxel in a mouse model of lung metastasis [25]. In 2005, a fullerene–paclitaxel conjugate was encapsulated in liposomes for aerosol therapy [36]. New nanovector colloidal formulations of paclitaxel without Cremophor®EL have been described in the literature such as nanoparticles [37], nanospheres [38], nanoemulsions [39] and linked to human albumin [40]. These formulations must be administered intravenously but none of these formulations have already been administered by inhalation. LNC, an original nanostructure vector hybrid between polymeric nanocapsules and liposomes, is the first nanocolloidal vector to deliver paclitaxel by aerosol compatible with therapeutic use in humans. Because PTX–LNC is a sustained-release drug nanocarrier and can be nebulised, the traditional limitations for the blood infusion paclitaxel route of administration – such as a low concentration into the lungs; insufficient drug delivery to the tumour cells, inducing cell resistance; and non-specific distribution associated with systemic toxicity – could be overcome with aerosols of PTX–LNC in lung malignancy patients. In addition, the LNC carrier acts as an inhibitor of the P-gp, which is a major mechanism of resistance to paclitaxel [41], allowing us to enhance the therapeutic potential of PTX.

To date, only a few non-liposomal vectors for conventional chemotherapy allow aerosol delivery. Tseng et al. presented gelatin nanoparticles with biotinylated epidermal growth factor set at the outside of the structure to allow the active targeting to the EGF receptor for lung cancer targeting [42]. These nanoparticles had a diameter of between 200 and 300 nm and could encapsulate cisplatin. A dry powder formulation of doxorubicin encapsulated in nanoparticles was described by Azarmi et al. [43]. This vector was produced by a method of polymerisation/emulsion and then dried. After being re-dissolved in deionised water, the particles had an average size of 173 ± 43 nm. The lipid nanocapsule is a new and original ready-to-use nanovector for aerosol delivery. As the LNC can encapsulate many different anticancer drugs such as paclitaxel or etoposide, this drug nanocarrier allows the testing of new pulmonary delivery strategies in lung malignancy treatment.

5. Conclusions

This study demonstrates the feasibility of an LNC paclitaxel aerosol. The characteristics of the aerosols are compatible with use in human medicine. A scaled-up procedure and a mid-term conservation procedure have been developed for preclinical and clinical studies. Animal studies are needed to determine the therapeutic potential of the aerosols of LNC dispersion.

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