International Journal of Pharmaceutics 379 (2009) 266–269

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

The adaptation of lipid nanocapsule formulations for blood administration in animals

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ARTICLE INFO

Article history:
Received 30 January 2009
Received in revised form 14 April 2009
Accepted 18 May 2009
Available online 23 May 2009

Keywords:
Lipid nanocapsule
Paclitaxel
Animal experimentation
Intravenous injections
Plants
Genetically modified
Drug storage

ABSTRACT

In many cell-culture and animal models, the therapeutic effects of the entrapped drugs in lipid nanocapsules (LNCs) were preserved with low toxicity. These results allow foreseeing further preclinical efficiency and toxicity studies in animals. In this article, preliminary studies were performed to check the genetically modified organism (GMO) status of the LNCs components and to determine the effects of the acidity of the LNCs dispersions in acid–base balance in rats. Then, several freezing protocols to store paclitaxel-loaded LNCs dispersions for a 6-month period were compared. Results indicate that the Lipoïd® S75-3 could not be certified GMO-free. The same soya bean lecithin certified to be GMO-free permitted to produce LNCs with expected characteristics. The blood administration of blank LNCs dispersions in rats induced no modifications of blood acidity, but a significant decrease of the base excess was observed. Injections of LNCs dispersions in animals might induce iatrogenic acidosis. We finally demonstrated that the best protocol to store LNCs dispersion for a 6-month period is by freezing in liquid nitrogen. This protocol minimized the characteristics modifications and interrupted the drug-release phenomenon. These original data are expected to prepare of LNCs dispersions well adapted for i.v. administration in animals.

1. Introduction

Lipid nanocapsules (LNCs) are the result of original, solvent-free technology allowing the production of new drug nanocarriers able to transport soluble, lipophilic drugs (Heurtault et al., 2002). Many different drugs have been encapsulated, such as amiodarone (Lamprecht et al., 2002), ibuprofen (Lamprecht et al., 2004), tripentone (Malzert-Freon et al., 2006), etoposide (Lamprecht and Benoit, 2006), ferrocenyl diphenol (Allard et al., 2008), docetaxel (Khalid et al., 2006) and paclitaxel (Garcion et al., 2006; Peltier et al., 2006).

In many cell-culture and animal models, the therapeutic effects of the entrapped drugs were preserved with a low level of toxicity. For example, paclitaxel-loaded LNCs demonstrated a significant anti-tumoral effect on cell-culture and human tumour models in mice in comparison with the commercially available formulation Taxol® (Garcion et al., 2006; Lacœuille et al., 2007). These results are attributed to the intrinsic properties of LNCs that reduce potential cytotoxic effects of paclitaxel by increasing the amount of paclitaxel in the tumour due to enhanced permeability and retention (EPR), the sustained release of paclitaxel (Lacœuille et al., 2007), and the inhibition of P-glycoprotein (P-gp) (Garcion et al., 2006). These results allow us to foresee further preclinical efficiency and toxicity studies in animals.

Captex® 8000 and Lipoïd® S75-3, two major components of the LNCs, are extracted from coconut, palm and soya bean oils, respectively. The systematic research of the presence of genetically modified organisms (GMOs) in all the components of the LNCs was needed prior to undertaking animal toxicity studies. Additionally, it has been noted that LNCs dispersions are acidic, but no data are available concerning the impact of single LNCs dispersion via blood injection on the acid-basic blood balance in animals. It is important to have this information before providing repeated injections in animals during toxicological screening. Finally, the best protocol for the storage of paclitaxel-loaded LNCs dispersions for preclinical studies is not currently known. Three 6-month freeze-storage protocols for paclitaxel-loaded LNCs were set up and compared.

2. Materials and methods

2.1. Materials

Captex® 8000 (glyceryl tricaprylate from coconut and palm oils) and Solutol® HS15 (polyglycol ester of 12-hydroxystearic acid and polyethylene glycol) were purchased from Abitec Corp

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doi:10.1016/j.ijpharm.2009.05.033

(Columbus, OH, USA) and BASF (Ludwigshafen, Germany), respectively. Lipoïd® S75-3 and Lipoïd® P75-3 (both soya bean lecithin at 69% phosphatidylcholine) were purchased from Lipoid GmbH (Ludwigshafen, Germany). 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). Ministar 0.20 μm high-flow filters were purchased from Sartorius AG (Goettingen, Germany). CRYO.S cryotubes from Greiner Bio.one were used (Frickenhausen, Germany).

2.2. LNCs formulation

The study was performed using 55 nm-diameter LNCs prepared according to an original method described elsewhere (Heurtault et al., 2002). 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 at 90 °C under magnetic stirring. Three cycles of heating and cooling between 90 °C and 70 °C were carried out before fast cooling was induced at 78 °C by adding 13.2 mL of 0 °C deionised water. Paclitaxel powder was first dissolved in Captex® 8000 at 1.8% (w/w) (i.e. 21.6 mg in 1.2 g) under magnetic stirring and heated at 50 °C for 30 min to form the oily phase. Then, the drug-loaded LNCs were manufactured according to the aforementioned process described.

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 SA., Worcestershire, UK) after filtration through a 0.20 μm Sartorius filter.

2.4. Drug payload and encapsulation efficiency

Paclitaxel-loaded LNCs were filtered through a 0.20 μm Sartorius filter before using a validated protocol to measure the payload and encapsulation efficiency by using a high-performance liquid chromatography (HPLC) protocol before and after 6-month storage.

2.5. Genetically modified organism status of the LNCs components

The genetically modified organism (GMO) status of the LNCs components was assessed by requesting the suppliers of each component to provide a GMO statement.

2.6. LNCs dispersion acidity and an animal, single-dose injection study

pH measurements of LNCs dispersions were performed by calibrated micro-pH CRISON 2001 OSI (Paris, France). The rats (Sprague Dawley, 290–318 g females) were obtained from the Faculty of Medicine of Angers University and had access to water and food *ad libitum*, prior to the experimental procedure. During inhalational anaesthesia, the rats received a slow injection of 20 ml/kg blank LNCs dispersion in the tail vein (Diehl et al., 2001). An intracardiac puncture was performed before the injection and 15 min later to collect arterial blood in order to carry out a blood gas analysis with an Opit CCA analyser (Roche Diagnostics GmbH, Mannheim, Germany). The animals were sacrificed after the second blood puncture. The experimental protocol was approved by the *Pays-de-la-Loire* ethics committee on animal experiments under number 2008-4, and studies were completed in conformance with French law.

2.7. Storage protocols

Paclitaxel-loaded LNCs dispersions were stored in CRYO.S cryotubes; they were firstly frozen in liquid nitrogen for 12 h, and then maintained in liquid nitrogen or stored at either −80 °C or −20 °C for 6 months. A specific thawing protocol was applied to each batch: liquid nitrogen-stored LNCs were kept for 1 h at −80 °C, then for 1 h at −20 °C and then thawed at room temperature for 30 min; −80 °C-stored LNCs were kept for 1 h at −20 °C before thawing at room temperature for 30 min; −20 °C-stored LNCs were thawed at room temperature for 30 min. These paclitaxel-loaded LNCs dispersions were then analysed for size distribution, zeta potential, drug payload and cytotoxicity assays.

2.8. in vitro cytotoxicity studies

The *in vitro* cytotoxic activity levels of fresh LNCs and the three groups of thawed paclitaxel-loaded LNCs for NCI-H460 human lung cancer cells were determined using a growth-inhibition assay. The NCI-H460 human, large-cell lung carcinoma cell line was obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were cultured in 1,640 RPMI 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% foetal bovine serum (Lonza, Verviers, Belgium), 50 U/mL penicillin, and 50 mg/mL streptomycin (Sigma–Aldrich Co., Ayrshire, UK). The cells were routinely maintained at 37 °C in a humid atmosphere with 5% CO_2. The culture medium was replaced every 2 or 3 days and the cells were subcultured weekly using 0.25% trypsin-1 mM EDTA (Sigma-Aldrich Co., Ayrshire, UK). Tumur cells were seeded in 24-well plates at 50,000 cells/well. All assays were performed with exponentially growing cultures. After 72 h, a drug-containing medium was added. Each sample, with a dose-range from 10^{-3} to 100 nM of paclitaxel-encapsulated LNCs 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. After 4 h in culture, the cell viability was determined by measuring the absorbency level at 492 nm using a Multiskan Ascent microplate reader (Thermo Fisher Scientific Cergy-Pontoise, France). Because the culture medium was not translucent when the LNCs were added, the absorbency of wells containing the cells was subtracted from the absorbency 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.

2.9. Statistical analysis

The results are expressed as a mean ± SD. For the analysis of statistical significance, the Mann–Whitney *U*-test was applied with Bonferroni's correction. For the comparison of several groups, a Kruskal–Wallis test was applied with Bonferroni's correction. In all cases, $P < 0.05$ was considered to be the level of statistical significance.

3. Results

3.1. Genetically modified organism check

Based on the certificates provided by the suppliers, only the Lipoïd® S75-3 (soya bean lecithin at 69% of phosphatidylcholine) could not be certified GMO-free. This element was replaced by Lipoïd® P75-3, a soya bean lecithin certified by the supplier to be GMO-free. The replacement of this component did not affect the

Table 1
Characterisations of blank LNCs produced by using Lipoïd® S75-3 and Lipoïd® P75-3; experiments performed in triplicate.

	Mean particle size (nm)	Polydispersity index	Zeta potential (mV)	Volume (ml)
Formulation with Lipoïd® S75-3	50.4 ± 0.7	0.05 ± 0.01	−4.85 ± 0.55	15
Formulation with Lipoïd® P75-3	52.4 ± 0.5	0.05 ± 0.02	−5.76 ± 0.40	15
Mann–Whitney *U*-test	$P > 0.05$	$P > 0.05$	$P > 0.05$	–

Table 2
Results of a blood gas analysis of three rats before and 15 min after the i.v. administration of blank LNCs.

	Time (min.)	pH	pCO_2 (mmHg)	pO_2 (mmHg)	Base excess	Haemoglobin (g/l)	Hematocrit value (%)	Oxygen saturation (%)
Rat 1	0	7.39	49	95	3.5	10.2	31	94
	15	7.40	41	227	−0.3	11.9	36	100
Rat 2	0	7.48	34	174	1.8	11.3	34	99
	15	7.47	36	223	−2.8	12.0	35	98
Rat 3	0	7.47	43	150	5.9	14.4	43	100
	15	7.37	44	324	0.9	14.1	42	100

Table 3
Characterisations of paclitaxel-loaded LNCs after the 6-month storage protocols in comparison with fresh paclitaxel-loaded LNCs.

	Mean particle size (nm)	Polydispersity index	Zeta potential (mV)	Drug payload (mg/ml)
Fresh paclitaxel-loaded LNCs	53.4 ± 1.9	0.08 ± 0.02	−5.84 ± 0.87	1.65 ± 0.08
Paclitaxel-loaded LNCs stored for 6 months in liquid nitrogen	63.4 ± 1.3[a]	0.13 ± 0.02[a]	−6.61 ± 0.30	1.64 ± 0.08
Paclitaxel-loaded LNCs frozen in liquid nitrogen then stored for 6 months at −80 °C	65.6 ± 1.4[a]	0.14 ± 0.01[a]	−4.01 ± 0.41[a,c]	1.61 ± 0.07
Paclitaxel-loaded LNCs frozen in liquid nitrogen then stored for 6 months at −20 °C	105.0 ± 1.7[a,b]	0.21 ± 0.01[a,b]	−3.34 ± 0.25[a,d]	1.58 ± 0.09
Kruskal–Wallis test (between four groups)	$P = 0.005$	$P = 0.006$	$P = 0.006$	$P > 0.05$
Kruskal–Wallis test (between frozen groups only)	$P = 0.013$	$P = 0.009$	$P = 0.009$	$P > 0.05$

[a] $P = 0.025$ versus fresh paclitaxel-loaded LNCs (Mann–Whitney *U*-test).
[b] $P = 0.025$ versus the '−80 °C' or 'liquid nitrogen' groups (Mann–Whitney *U*-test).
[c] $P = 0.025$ versus the 'liquid nitrogen' group (Mann–Whitney *U*-test).
[d] $P = 0.025$ versus the '80 °C' group (Mann–Whitney *U*-test).

characteristics of a GMO-certified LNCs dispersion in comparison with LNCs dispersion produced with the Lipoïd® S75-3 (Table 1).

3.2. The effects of LNCs dispersion acidity on the rat acid–base balance

A blank formulation with a mean size of 50.4 nm had a pH of 5.93 ± 0.05. For the three rats, 20 ml/kg of this blank LNCs dispersion (2.9–3.1 ml), corresponding to the maximum recommended volume, was slowly injected into the lateral tail vein. Blood gas analysis results are presented in Table 2. During the procedure, no modifications of vital clinical signs or of the pCO_2 levels or the haemoglobin levels were observed. No clinically pertinent modifications of blood acidity were observed, but a significant decrease of the base excess by a mean value of 4.5 ± 0.6 mmol/l was constantly observed.

3.3. Storage protocols

Results of the characterisation of the paclitaxel-loaded LNCs after 6 months storage are presented in Table 3. Statistical analysis revealed significant differences in mean particle size ($P = 0.005$), the polydispersity index ($P = 0.006$) and the zeta potential ($P = 0.006$) between frozen and fresh paclitaxel-loaded LNCs groups. Each storage protocol induced a significant increase in the mean particle size and the polydispersity index in all the frozen LNCs groups ($P = 0.025$ versus fresh paclitaxel-loaded LNCs – Mann–Whitney *U*-test). A significant decrease of the zeta potential was observed in LNCs dispersions not stored in liquid nitrogen ($P = 0.025$ versus fresh paclitaxel-loaded LNCs – Mann–Whitney *U*-test). No modifications of the paclitaxel payload were observed. The specific statistical analysis of the frozen paclitaxel LNCs groups showed significant differences in mean particle size ($P = 0.013$), the polydispersity index ($P = 0.009$) and the zeta potential ($P = 0.009$). The mean particle size and the polydispersity index were significantly increased in the '−20 °C storage' group ($P = 0.025$ versus the '−80 °C' or 'liquid nitrogen' groups). Zeta potentials were significantly decreased in the

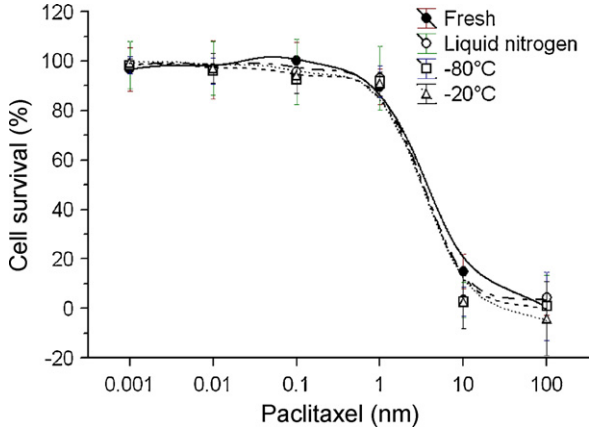

Fig. 1. The effects of paclitaxel-loaded LNCs stored for 6 months according to three different protocols (liquid nitrogen, −80 °C, and −20 °C) in comparison with fresh paclitaxel-loaded LNCs on NCI-H460 cell growth.

'−80 °C' group ($P = 0.025$ versus the 'liquid nitrogen' group) and in the '−20 °C' group ($P = 0.025$ versus the '80 °C' group). Cytotoxicity assays are presented in Fig. 1. The IC 50 values of paclitaxel for NCI-H460 cell lines were equal to 5.7 ± 0.2 nM, 5.3 ± 0.3, 5.2 ± 0.1 and 5.2 ± 0.1 for fresh, liquid nitrogen-stored, −80 °C stored and −20 °C stored paclitaxel-loaded LNCs ($P > 0.05$), respectively.

4. Discussion

In this study, three original topics concerning LNCs dispersions are studied. Firstly, potential contamination from GM soya bean in Lipoïd® S75-3 was unveiled. As the clinical output of GM products on human health are not known, and as numerous complementary studies for registration by regulatory agencies could be requested, as suggested by a U.S. Food and Drug Administration draft guidance (U.S. Food and Drug Administration, 2002), the replacement of this component was required. This step was easily reached by using a similar, GMO-free product from the same supplier. GMO-free LNCs dispersions, strictly similar to original ones, are now available for further studies. By using the GMO-free Lipoïd® P75-3 lecithin, we are guaranteed to formulate LNCs with constant-quality using GMO-free components only, and hence avoid supplementary safety studies.

Since repeated injections of LNCs dispersions in animals are used for this research, and since LNCs dispersions are known to be acidic, it is necessary to measure the pH levels of LNCs dispersions and to determine their impact on the acid–base homeostasis of animals. The pH level was below 6. LNCs administration in rats was carried out at the maximum possible volume during a slow injection to reveal any potential effects on the acid–base balance. During the experiment, no clinical modifications in the animals were observed. The absence of respiratory failure or internal haemorrhaging as indicated by the blood samples are well documented by unchanged levels of both pCO_2 and haemoglobin during the experiment. The results showed the absence of clinically significant blood acidity modification, but a notable diminution of the base excess was observed. We hypothesised that repeated injections of acid LNCs dispersions in animals might induce iatrogenic acidosis by exceeding acid intake levels for physiological control systems in animals as observed in humans (Lim, 2007). For this reason, the pH of the LNCs dispersions for i.v. administration needs to be buffered to reach a more physiologically-acceptable pH range. Studies to buffer LNCs dispersions are on going.

Because LNCs are sustained release drug carriers, storage methods must preserve their structure and also interrupt the drug-release phenomenon. Our research looked at freezing strategies because freezing seems easy to carry out and avoids complex manipulations of anticancer-loaded nanocarriers. Fast cooling with liquid nitrogen followed by conservation in liquid nitrogen or in a freezer at very low (−80 °C) or low (−20 °C) temperatures were compared. Standardised protocols were applied to the LNCs dispersions to allow them to thaw out. Although no significant modifications of the drug payload and the IC 50 values were observed, some modifications of the LNCs structural characteristics were noticed. Firstly, each storage protocol induced an increase of the mean particle size and of the polydispersity index. Modifications were low for the liquid nitrogen and the −80 °C groups, but significantly higher for the LNCs stored at −20 °C. Secondly, a modification of the zeta potential was observed when LNCs were not stored in liquid nitrogen and was, interestingly, inversely correlated with the storage temperature (linear correlation coefficient = 0.98). We conclude that the 6-month freezing storage protocol in liquid nitrogen induces the lowest number of modifications to the paclitaxel-loaded LNCs dispersions. The two other protocols induced greater modifications of LNCs characteristics, especially the zeta potential; this could modify their biodistribution in animals or even trigger toxicity. Complementary studies are needed to verify that the freezing storage protocol in liquid nitrogen do not modify future LNCs dispersions compatible with repeated i.v. administration.

5. Conclusions

In this study we made two important adaptations of the LNCs standard formulation. Firstly, we replaced a potentially GMO-polluted component with its GMO-free equivalent. Secondly, we demonstrated that the acidity of LNCs dispersions can induce physiological disorders by acidifying blood after a single i.v. administration. Before using repeated injections in animals, the acidity of the dispersions should be buffered to avoid metabolic acidosis. Finally, we demonstrated that the best protocol for a 6-month storage period of paclitaxel-loaded LNCs is to freeze them in liquid nitrogen which avoids any significant modifications to their main characteristics. These results are expected to prepare of LNCs dispersions well adapted for i.v. administration in animals.

Acknowledgements

The authors would like to thank Pierre Legras and Jérôme Roux (Angers University, Faculty of Medicine, Angers, France) for help in the animal studies.This study was supported by grants from the Comité Départemental du Maine-et-Loire de la Ligue Contre le Cancer, the Cancéropôle Grand Ouest and the Régions Bretagne, Centre, Pays de la Loire et Poitou-Charentes.

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