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Research Paper

Toxicological Study and Efficacy of Blank and Paclitaxel-Loaded Lipid Nanocapsules After *i.v.* Administration in Mice

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Purpose. Lipid nanocapsules (LNCs) are solvent-free drug nanocarriers permitting entrapment of paclitaxel and increasing its antitumoural effect in animal models after *i.v.* injection. The tolerance and efficacy of LNCs after repeated dose *i.v.* administration were assessed in mice. The maximum tolerated dose (MTD) and 50% lethal dose (LD50) were studied.

Methods. Paclitaxel-loaded LNC formulation was given *i.v.* at the dose of 12 mg/kg per day for 5 consecutive days in comparison with blank LNCs and saline. Histological examination, complete blood counts and biochemical quantification were performed after a recovery of 7 days. Growth of NCI-H460 subcutaneous xenografts in nude mice receiving one of the aforementioned schedules was assessed. MTD and LD50 were determined by Irwin test.

Results. No mortality was observed in repeated injections studies. Histological studies revealed no lesions and no accumulation of lipids. Blood studies were normal. The tumoural growth was significantly reduced in the group treated by paclitaxel-loaded LNCs. The MTDs/LD50s of Taxol®, paclitaxel-loaded LNCs and blank LNCs were 12/19.5, 96/216 and above 288/288 mg/kg, respectively.

Conclusions. This study demonstrates that a five-day *i.v.* injection schedule of paclitaxel-loaded LNC dispersions induces no histological or biochemical abnormalities in mice and improves paclitaxel efficacy and therapeutic index in comparison with Taxol®.

KEY WORDS: biocompatibiliy; controlled drug release; drug delivery; *in vivo* test; nanoparticle.

INTRODUCTION

Taxol® chemotherapy has provided a major therapeutic improvement for several solid tumours, such as ovarian, breast and non-small cell lung cancers (1). Paclitaxel, the first drug of the taxane class, has resulted in observations of tumour reduction in refractory cases. Microtubule assembly inducing antiproliferative, antiangiogenic, and antimetastatic effects has been proposed to explain the observed therapeutic effects (2,3). Nevertheless, paclitaxel is water-insoluble and has a low solubility in most pharmaceutically acceptable solvents. In Taxol®, the first paclitaxel formulation used in clinical practice, paclitaxel, is solubilised by a surfactant in an organic medium, Cremophor®EL (polyethoxylated castor oil), and dehydrated ethanol in a 1:1 (*vol/vol*) ratio. Cremophor®EL is an excipient with notorious effects on humans, such as life-threatening anaphylaxis (4–6), hyperlipidaemia (7), abnormal lipoprotein patterns (8,9), the aggregation of erythrocytes (10), and peripheral neuropathy (11–13). Some studies indicate that Cremophor®EL may trigger complement activation (14–16). In rats, Cremophor®EL may induce adverse haemodynamic effects (17). Because of these numerous side effects, Cremophor®EL-free formulations of paclitaxel have been developed and are expected to show less toxicity for patients during chemotherapy treatment.

Different strategies to formulate paclitaxel have been developed, such as polymers (18), prodrugs (19), microemulsions (20), nanoemulsions (21), micrometer-size porous particles (22), nanoparticles (23), nanospheres (24), liposomes (25) and an albumin-stabilised nanoparticle formulation (26). To date, only the last solvent-free formulation, Abraxane®, has demonstrated better efficacy and an alleviation of toxic effects than Taxol® during a Phase III study in metastatic breast cancer patients (27). This new formulation was approved by the Food and Drug Administration (FDA) for clinical use in February 2007. Encouraging Phase I and II clinical studies have been published for solid tumours as well as for non-small cell lung cancer (28) and recurrent ovarian, peritoneal, or fallopian tube cancer (29).

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The albumin-bound paclitaxel nanoparticle (NAB-paclitaxel) has an original tumoural concentration mechanism *via* an albumin receptor, and is analogous to the opening of a "trap door" on the endothelial cell wall within neo-vascularisation (30). This nano-technology eliminates the use of toxic excipients like Cremophor®EL and thereby allows an increase in the quantity of paclitaxel administered to patients, producing reasonable toxicity effects (31).

Lipid nanocapsules (LNCs) represent nanocarriers designed to encapsulate lipophilic drugs, such as paclitaxel, without using organic solvents. An original phase-inversion process allows the production of nanocarriers ranging from 25 to 100 nm in a saline solution (32). The carrier had a unimodal size distribution and a low polydispersity index. Structurally, the lipophilic drug is solubilised into the central lipid core, which is surrounded by a membrane of lecithins and pegylated hydroxystearate (HS-PEG) in order to provide stealth properties (33). At human body temperature, the core of the LNC is liquid, whereas the membrane is rigid. Numerous drugs can be encapsulated in LNCs: amiodarone (34), ibuprofen (35), tripentone (36), etoposide (37), ferrocenyl diphenol (38), docetaxel (39), and paclitaxel (40,41). In the field of oncology, etoposide- and paclitaxel-loaded LNCs have shown higher cytotoxicity effects than free drugs after systemic administration; this has been explained by the properties of LNCs to concentrate into tumoural tissues due to the Enhanced Permeability and Retention (EPR) effect and sustained release of paclitaxel and P-glycoprotein (P-gp) inhibition by Solutol® HS15 (37,40).

To date, no data about the toxicity of LNCs administered in blood are available in animals. In addition, no study has been realised to test the efficacy of repeated *i.v.* injections of paclitaxel-loaded LNCs in xenografts models. The aim of this study is to carry out the first *in vivo* toxicological studies after *i.v.* injections of blank and paclitaxel-loaded LNC dispersions in Swiss mice. First, the toxicity, survival, behaviour modifications, gross and microscopic anatomy, complete blood count, blood biochemical assays (liver and renal excretion functions), and C-Reactive protein dosage were studied in Swiss mice after the repeated blood injection of blank and paclitaxel-loaded LNCs. Second, the efficacy of the same schedule of paclitaxel-loaded LNC dispersions was assessed in a NCI-H460 subcutaneous xenografts model in nude mice. Third, since the LNCs are composed of an oily core, the potential accumulation of lipids in the lungs, liver, spleen and kidneys of Swiss mice after the repeated blood-administration of LNCs was studied in histological slices. Finally, the maximum tolerated dose (MTD) and 50% lethal dose (LD50) of blank and paclitaxel-loaded LNCs from a single administration were determined.

MATERIALS AND METHODS

Materials

Lipoïd® S75-3 (genetically-modified, organism-free, soybean lecithin at 69% of phosphatidylcholine), Captex® 8000 (glyceryl tricaprylate) and Solutol® HS15 (polyethyleneglycol ester of 12-hydroxystearic acid and polyethylene glycol) were obtained from Lipoid GmbH (Ludwigshafen, Germany), Abitec Corp (Colombus, OH, USA) and BASF (Ludwigshafen, Germany), respectively. Taxol® 6 mg/mL solutions and paclitaxel powder were supplied by Bristol-Myers Squibb (Rueil-Malmaison, France) and AMPAC Fine Chemicals (Rancho Cordova, CA, USA), respectively. Distilled water and sodium chloride were purchased from Cooper (Melun, France) and Prolabo VWR International (Fontenay-sous-Bois, France), respectively. Ministar® 0.20 µm high-flow filters from Sartorius AG (Goettingen, Germany) were used. CRYO.S cryotubes were purchased from Greiner Bio.one (Frickenhausen, Germany). The 4% phosphate-buffered formalin was provided by Labonord (Templemars, France). Animal injections were performed with 1 ml Ultra-Fine, 29-gauge insuline syringes (Plastipack, BD, Erembodegem, Belgium).

LNC Formulation

The study was made on 55 nm diameter LNCs prepared according to a scaled-up protocol that respects the original process (32) and allows the production of standard batches for *in vivo* studies (32,42,43). Briefly, Captex® 8000, Lipoïd® S75-3, Solutol® HS15, NaCl and water (12 g, 672 mg, 10 g, 733 mg, and 18 g, respectively) were mixed and heated under magnetic stirring up to 90°C. Three cycles of progressive heating and cooling between 90°C and 70°C were then carried out. Thermal exchanges were optimised by bathing in water at 95°C and at room temperature. This step was followed by an irreversible shock, induced by dilution with 112 mL of 0°C, deionized water added to the mixture when it was at 78°C. Magnetic stirring was applied to the suspension of LNCs for 5 min at room temperature, and molar sodium hydroxide and NaCl were added (50 µl and 110 mg, respectively). The dispersion was filtered through a 0.20 µm Ministar® filter into a CRYO.S cryotube.

For the formulation of paclitaxel-loaded LNCs, 1.8% w/w of paclitaxel powder was first solubilised in Captex® 8000 (i.e. 216 mg in 12 g) under magnetic stirring and heating at 50°C for 30 min. The above-cited procedure was then applied to this mixture.

According to the same process, high-concentration formulations were prepared. The complete process was applied by mixing Lipoïd® S75-3, Solutol® HS15, NaCl and water (12 g, 672 mg, 10 g, 250 mg, and 18 g, respectively). The final dilution was made with 38 ml of deionized water at 0°C added at 88°C. Magnetic stirring was applied to the suspension of LNCs for 5 min at room temperature, and molar sodium hydroxyde was added (50 µl). The dispersion was filtered through a 0.20 µm Ministar® filter into a CRYO.S. cryotube. For the formulation of paclitaxel-loaded LNCs, 2.0% w/w of paclitaxel powder was first solubilised in Captex® 8000 (i.e. 240 mg in 12 g) under magnetic stirring and heating at 50°C for 30 min. The above-cited procedure was then applied to this mixture.

LNC Conservation Protocol

The CRYO.S cryotubes that had previously been filled with LNC dispersions were frozen in liquid nitrogen. The thawing protocol consisted of thawing the CRYO.S cryotubes at room temperature for 30 min.

LNC Characterisation and Drug Payload

Size distribution, the polydispersity index and the zeta potential measurements of thawed LNC dispersions were characterised by photon-correlation spectroscopy performed by using a Malvern Zetasizer®, Nano Series DTS 1060 (Malvern Instruments S.A., Worcestershire, UK). In order to determine the drug payload, the paclitaxel-loaded LNC dispersions were filtered by 0.20 μm Ministar® high-flow filters. Paclitaxel concentrations and encapsulation efficiency were measured in the supernatant by High Performance Liquid Chromatography (HPLC) in triplicate experiments according to a described protocol (42).

Animals

Ninety-eight female, Swiss mice (7 weeks old, 20–22 g) and 27 male nude (nu/nu) athymic mice (6 weeks, 18–22 g) were obtained from Charles River Laboratories (L'Arbresle, France). The mice had access to water and food *ad libitum*, prior to and during experimental procedures. The animals were acclimatised for 7 days before being included in the studies. Both LNC formulations and saline serum were slowly injected in the lateral tail vein during short, inhalational anaesthesia induced by isoflurane. Weight loss, the aspect of the fur, respiratory rate, behaviour and responses to normal stimuli were evaluated daily according to the toxicity scale presented in Table I. The animals were put down if the daily score was greater than 8. The research adhered to the "Principles of Laboratory Animal Care" (NIH publication #85-23, revised in 1985). The experimental protocols were approved by the Pays-de-la-Loire ethics committee on animal experiments (number 2008-7), and the studies were completed in compliance with French law.

Repeated-Dose Toxicity Studies

Thirty-five mice received either an intravenous injection of saline serum, a standard, blank LNC dispersion, or a standard paclitaxel-loaded LNC dispersion for 5 consecutive days. The injected volume of the standard, paclitaxel-loaded LNC dispersions corresponds to the dosage of 12 mg/kg/day (146 to 160 μl per injection). The volume of saline serum and standard blank LNCs was equal to the volume of the standard paclitaxel-loaded LNC dispersion.

Histological Study

Groups of six, six, and three Swiss mice received paclitaxel-loaded LNCs (P group), blank LNCs (B group) and saline serum (S group) for 5 days, respectively. After a 7-day recovery period, the Swiss mice were sacrificed by lethal, inhalational anaesthesia. Macroscopic and histological examinations of the lungs, liver, spleen and kidneys, fixed in 4% phosphate-buffered formalin, were performed. The organs were embedded in paraffin, and 5-μm sections were stained with haematoxylin-eosin-saffron. A new histopathological score was designed to assess lung inflammation in four lung sectors: peribronchiolal, perivascular, interstitial and alveolar. For each sector, the histopathological aspect was scored on a scale from 0 to 3:0 (no change: normal aspect), 1 (mild inflammation: dispersed mononuclear leukocytes), 2 (moderate inflammation: presence of 1 to 3 nodules of mononuclear leukocytes), 3 (severe inflammation: more than 3 nodules of mononuclear leukocytes). The final score was the sum of the highest scores observed in each sector of the lungs. The pathologist was unaware of the treatment received by the mice.

Lipid Accumulation

Two groups of three Swiss mice received either a blood injection of saline serum or blank LNC dispersions at the dosage of 12 mg/kg/day of paclitaxel-loaded LNCs for 5 days. After a recovery period of one week, the mice were sacrificed by lethal anaesthesia. The liver, spleen and kidneys were removed and frozen in liquid nitrogen before staining for fat by Oil Red O.

Biological Study

Groups of five, five, and four Swiss mice received paclitaxel-loaded LNCs (P group), blank LNCs (B group) and saline serum (S group) for 5 days, respectively. After one week of recovery, the mice were sacrificed by lethal, inhalational anaesthesia. Blood samples were collected by intracardiac puncture on ethylene-diamine-tetraacetic acid (EDTA) tubes for a complete blood count and a haematocrit count. Analyses were performed in the Haematology Ward of the Academic Hospital of Angers with an XE-2100 haematology analyser (Sysmex). After centrifugation of whole blood, serums were obtained for the biological analysis of natremia, kaliemia, chloremia, the alanine aminotransferase (ALAT) rate, the total bilirubin rate and creatinine levels. These analyses were carried out at the Biochemistry Ward of the Academic Hospital of Angers on a Modular P® (Roche diagnostics). The C-Reactive protein was quantified in the serum by using a Demeditec C-Reactive protein ELISA assay according to the supplier's instructions.

Table I. Toxicity Scale. The Sum of Each Criterion Allows the Calculation of the Daily Score for Each Mouse

	No point	1 point	2 points	3 points
Loss of weight	<5%	5–12%	13–20%	>20%
Aspect of the fur	Normal	Mild alteration	Moderate alteration	Severe alteration
Respiratory rate	Normal (100/minute)	Increase or decrease less than 10%	Increase or decrease less than 20%	Increase or decrease less than 30%
Behaviour	Normal	Mild modification	Moderate modification	Severe modification
Responds to normal stimuli	Normal	Mild modification	Moderate modification	Severe modification

Single-Dose Toxicity Study

The maximum tolerated dose (MTD) and 50% lethal dose (LD50) were assessed for high-concentration blank LNCs and paclitaxel-loaded LNCs in comparison with Taxol® (diluted at 1.2 mg/ml in saline serum) and saline serum in accordance with Irwin test (44,45). Sixty-three Swiss mice were included in this study. The MTD is defined as the highest possible dose resulting in no animal deaths, less than 20% weight loss of the control animals and no particular changes in general signs (Table I). Animals showing a weight loss exceeding 20% were sacrificed for ethical reasons. The LD50 value is the amount of LNC dispersion that induces the death of 50% of the animals according to the Wilcoxon method. Tested doses were 12, 18 and 24 mg/kg for Taxol® and 12, 24, 48, 96, 192 and 288 mg/kg for the high-concentration, paclitaxel-loaded LNCs. Equivalent volumes of saline serum and blank, high-concentration LNCs were used in corresponding groups. The animals were observed daily. After a survey period of 28 days, the Swiss mice were sacrificed by lethal anaesthesia. The research adhered to the "Principles of Laboratory Animal Care" (NIH publication #85-23, revised in 1985). The experimental protocol was approved by the Pays-de-la-Loire ethics committee on animal experiments (number 2009-5), and the study was completed in compliance with French law.

Efficacy Study

Tumour Cell Line

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% CO2 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).

Xenograft Model

Nude mice were injected subcutaneously in the right flank with 0.1 ml of cell suspension containing 1×10^6 NCI-H460 cells. Treatments were started when tumour in the nude mice reached a tumour volume of 100 mm^3. This day was designated Day 1 and defined as the day treatments started after random division of the mice into 3 groups of 9 mice. At Days 1, 2, 3, 4 and 5, a daily *i.v.* of the paclitaxel-loaded LNCs, Taxol® or saline serum was administered intravenously through the tail vein. The dosage of paclitaxel was 12 mg/kg/day (150 to 220 µl per injection) for paclitaxel-loaded LNCs and Taxol®. Before animal injections, Taxol® solution was diluted in saline serum at a final concentration of 1.2 mg/ml. The volume of saline serum was equal to the volume of the paclitaxel-loaded LNC dispersion calculated aforementioned.

In Vivo Antitumour Activity

Mice were observed daily, and body weight measurements and signs of stress (*e.g.,* lethargy, ruffled coat, and ataxia) were used to detect toxicities. Animals with ulcerated tumours or whose tumours exceeded 600 mm^3 were euthanized for ethical considerations. Electronic calliper (Mitutoyo Corp., Kawasaki, Japan) measurements of tumours were converted into mean tumour volume (TV) in mm^3 using the following formula: 1⁄2 [length (mm)]×[width (mm)]^2. Each tumour volume at Day 8 was expressed as relative tumour volume (RTV) according to the formula: $RTV=TV_8/TV_1$, where TV_8 is the tumour volume at Day 8, and TV_1 is the tumour volume at Day 1. Tumour regression (T/C%) at Day 8 was determined by calculating RTV according to the following formula: T/C%=100×(mean RTV of the treated group)/(mean RTV of control group). After sacrifice, the tumours were excised and fixed in 4% phosphate-buffered formalin and embedded in paraffin for histological control.

Statistical Analysis

The results are expressed as the mean ± SD. Multiple comparisons were made using Kruskal-Wallis's one-way analysis of variance with Bonferroni's correction (Statistica 8.0; Statsoft Inc., Maisons-Alfort, France). For the statistical significance analysis, a Mann-Whitney test (non-parametric) with Bonferroni's correction was used to measure statistical significance between two treatment groups. In all cases, $P<0.05$ was considered to be significant.

RESULTS

Physico-chemical Properties of the LNCs

Preclinical batches of standard, blank and paclitaxel-loaded LNCs were formulated, stored in liquid nitrogen and thawed before use. Monomodal particle size distributions were 65.7±1.8 nm and 67.5±0.5 nm (mean ± SD) for blank and paclitaxel-loaded LNCs, respectively, with a narrow distribution (polydispersity index <0.1) (Fig. 1). The zeta potentials for blank and paclitaxel-loaded LNC dispersions were equal to −5.66±0.19 mV and −5.26±0.49 mV, respectively. After thawing, the payload and the encapsulation efficiency were equal to 1.65 mg/ml and 100.5±3.0%, respectively. For both batches, the acidity and osmolarity were 7.3±0.1 pH and 267±3 mOsm/kg, respectively.

High-concentration formulations of preclinical batches of blank and paclitaxel-loaded LNCs were obtained, stored in liquid nitrogen and thawed before use. Monomodal particle size distributions were 61.7±1.2 nm and 69.8±0.3 nm (mean ± SD) for blank and paclitaxel-loaded LNCs, respectively, with a narrow distribution (polydispersity index <0.2). The zeta potentials for blank and paclitaxel-loaded LNC dispersions were equal to −5.96±0.37 mV and −7.61±0.60 mV, respectively. After thawing, the payload and the encapsulation efficiency were equal to 2.99 mg/ml and 99.1±4.0%, respectively. For both batches, the acidity and osmolarity were 7.2±0.2 pH and 333± 2 mOsm/kg, respectively.

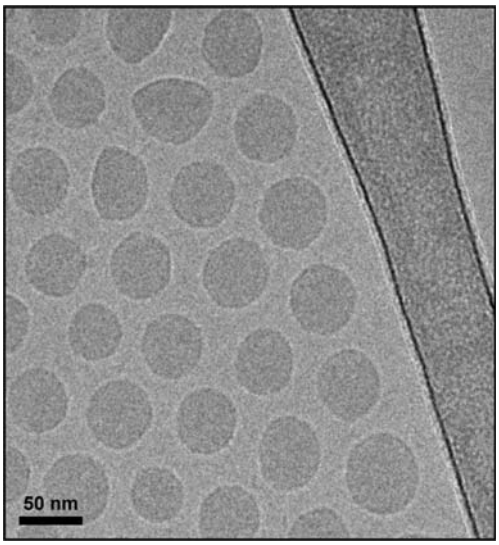

Fig. 1. Cryo-TEM image of the LNCs.

Repeated-Dose Toxicity Studies

No mortality was observed during these studies.

Histological Study

At Day 1, the day of the first injection, mean weights were 20.3±0.4 g, 20.8±0.4 g and 20.5±0.6 g in groups P, B and S, respectively (P>0.05). During the study, the mean body weight of the animals of all groups increased and were similar at Day 12 (P > 0.05). The behavioural study provided mean, daily toxicity scores in all groups of 0.0±0.0 at Days 0, 8 and 12. At Day 5, the scores were 0.0±0.0 in the S group and 0.2± 0.2 in both the P and B groups (P>0.05). During autopsy, no abnormalities were observed. The mean weights of the organs of interest are presented in Table II. No statistical differences were found. Histological studies revealed that the livers, spleens and kidneys were normal. The anatomic lung sections and global histopathological scores for lungs are presented in Table III. No statistical differences were observed.

Lipid Accumulation

The weight of the Swiss mice was not significantly different in the two groups, and the toxicity scores were 0 throughout the study. No lipid accumulation was observed in the liver, spleen or kidneys.

Biological Study

The mean weights of the mouse groups remained similar during the experiment. No toxicity was observed. Complete blood count results are presented in Table IV. Natremia, kaliemia, chloremia, ALAT, total bilirubin, serum creatinine and C-Reactive protein in serum data are presented in Table V. No statistical differences were observed.

Single-Dose Toxicity Study

No mortality was observed with saline serum. No mortality nor toxicity were observed after blood injection of blank LNCs. This result indicates that the blank LNC drug nanocarrier is not the limiting factor of toxicity. Total lipid amount that could solubilise up to 288 mg/kg of paclitaxel is not toxic. The MTD of blank LNCs was equal to or greater than 288 mg/kg, and the LD50 was not reached. For Taxol®, the MTD and LD50 were 12 mg/kg and 19.5 mg/kg, respectively. The MTD and LD50 of paclitaxel-loaded LNCs were 96 mg/kg and 216 mg/kg, respectively. Detailed results are presented in Table VI.

Antitumour Efficiency Study

Twenty-seven nude mice were randomised in three groups: a control group received saline serum (S group) alone, a group received Taxol® (T group) and a group received standard paclitaxel-loaded LNCs (N group). At Day 1, the mean size tumours were equal to 102.0±5.4 mm^3, 108.1±16.6 mm^3 and 105.7±7.4 mm^3 for S, T and N groups, respectively (P>0.05). During the study, no death and no sign of stress were observed. Some mice lost up to 20% weight in groups T and N. No tumour size regression was observed in S group during the study. A reduction of the tumoural volume superior to 20% was observed in three and six mice in T and N groups, respectively. The mean tumoural volume regressions were equal to 25.3%±4.0 and 48.5%±25.8 in T and N groups, respectively. Mean tumour growths per groups from Day 1 to Day 8 were presented in Fig. 2. At Day 8, the mean tumoural volume was equal to 423±42 mm^3, 277±19 mm^3* and 167±34 mm^3*,** in the S, T and N groups, respectively (*P<0.04 *vs* group S ; **P<0,04 *vs* T, U tests with Bonferroni's correction). At Day 8, the T/C% was equal to 64%‡ and 38%‡‡ in T and N groups in comparison to the S group, respectively ($^{\ddagger}P$<0.04 *vs* S group; $^{\ddagger\ddagger}P$<0,05 *vs* T, U tests with Bonferroni's correction). The median time to reach a tumour volume superior or equal to 600 mm^3 was 11, 16† and 20†,†† days in groups S, T and N, respectively ($^{\dagger}P$<0.04 *vs* group S ; $^{\dagger\dagger}P$<0,04 *vs* T, U tests with Bonferroni's correction). Survival curves (until tumours exceeded 600 mm^3) were presented in Fig. 3.

Table II. Mean Weight of the Organs of Interest in Milligrammes in S (Saline Serum), B (Blank LNCs) and P (Paclitaxel-Loaded LNCs) Groups. Mean Values ± SD are Presented

	Liver	Spleen	Lungs	Kidneys
S group (n=3)	1335.0±213.5	97.7±22.8	228.7±16.6	385.7±28.7
B group (n=6)	1431.8±66.6	117.2±8.7	253.8±10.4	383.0±38.6
P group (n=6)	1315.0±53.0	91.8±5.0	204.3±10.6	354.3±32.0
Kruskal-Wallis test	NS (P=0.064)	NS	NS	NS

Table III. Histopathological Scores by Lung Anatomic Sectors in S (Saline Serum), B (Blank LNCs) and P (Paclitaxel-Loaded LNCs) Groups. Mean Values ± SD are Presented

	Peribronchial sector	Perivascular sector	Interstitial sector	Alveolar sector	Global score
S group (n=3)	1.0±0.0	1.3±0.3	1.0±0.0	0.0±0.0	3.3±0.3
B group (n=6)	1.0±0.2	0.8±0.2	0.5±0.2	0.0±0.0	2.3±0.3
P group (n=6)	1.5±0.2	1.0±0.0	0.7±0.2	0.0±0.0	3.3±0.4
Kruskal-Wallis test	NS	NS	NS	NS	NS

DISCUSSION

As with 40% of new medical drugs, paclitaxel is a highly lipophilic drug that can not be solubilised in water without an excipient. For the market formulation Taxol®, the excipients are a 1/1 (*vol/vol*) mixture of dehydrated ethanol and Cremophor®EL. Cremophor®EL is a polyoxyethylene castor oil derivative that can induce life-threatening events caused by histamine release, in spite of medical premedication with corticoids, H1 and H2 antihistamine drugs. Even if paclitaxel is solubilised with Cremophor®EL and ethanol, crystals of paclitaxel may form during perfusion requiring 0.22 μm filtration during administration. For these reasons, less toxic formulations of paclitaxel are needed. LNCs are drug nanocarriers able to entrap many hydrophobic drugs such as paclitaxel. The structure of this vector is unique, composed of an oily core (where the drug is solubilised), limited by lecithin surfactants, and surrounded by polyethylene-glycol chains. Paclitaxel-loaded LNCs have been widely studied over the last few years and have demonstrated an improvement of paclitaxel efficacy on cell cultures and tumour-bearing animal models in comparison with Taxol® (40,46). To date, no toxicological data for LNC vectors are available. In this study, we present the first toxicity study of an *i.v.* injection schedule of both blank and paclitaxel-loaded LNC dispersions in Swiss mice as proposed by the International Life Sciences Institute Research Foundation/Risk Science Institute (ILSI RF/RSI) Nanomaterial Toxicity Screening Working Group (47). We started the toxicological evaluation with the administration schedule of paclitaxel-loaded LNCs at the dosage of 12 mg/kg/day of paclitaxel over five days in mice; this corresponds to the dosage of 175 mg/m^2 every three weeks of Taxol® in humans used for a cycle of chemotherapy (48). Blank and paclitaxel-loaded LNCs were tested against saline serum to discriminate between specific LNC toxicity (saline serum *vs* blank LNCs) and paclitaxel-induced tissue lesions (blank LNCs *vs* paclitaxel-loaded LNCs). Studies were performed with marketable, insurance-quality, standard batches of LNCs, well-characterised for mean particle size, size distribution, zeta potential, sterility and pyrogenicity (42). The LNC conservation protocol was studied and did not modify the LNC structure, drug payload, or cytotoxic effect (43). As the LNCs were injected just after thawing into animals, this procedure lets us know the precise characteristics of LNCs, which is a key point in order to describe the *in vivo* toxicity and efficacy of LNC dispersions.

At the dose of 12 mg/kg per injection per day for 5 consecutive days for paclitaxel-loaded LNCs (or the corresponding volume for blank LNCs), no mortality, behaviour modification or weight loss were observed after a recovery period of one week. Gross and histological analysis of vital organs such as the liver, spleen, kidneys and lungs revealed no abnormalities in comparison with blank LNCs and serum groups. A new histopathological score designed to assess lung inflammation showed no inflammatory reaction in four lung sectors. As with many colloid vectors, lipid nanocapsules have been found in the liver, spleen and kidneys after intravenous injection in rats (49). This distribution is driven by the rapid clearance from systemic circulation by macrophages of the reticulo-endothelial system in the liver and spleen (50). This biodistribution is typical of the passive targeting of LNCs *in vivo* and can trigger lesions in these organs (49,51). In our study, histological examinations of these organs where both paclitaxel-loaded LNCs and blank LNCs were sequestrated showed no tissue lesions or lipid accumulation. Our study showed a tendency for increases of the weight of the liver and the ALAT in serum in paclitaxel-loaded LNC groups *vs* the blank LNC dispersion group and serum group. However, these results were not statistically significant and could suggest that liver tolerance may be the limiting step. To the contrary, no effect was observed on complete blood counts, indicating the absence of a myelosupressive effect of LNCs. Complement system activation and the macrophage uptake of LNCs (with a range of 20–100 nm) has been previously studied (33). The results indicated that the consumption of CH50 units with LNC dispersions was very low in parallel with positive controls (polymethyl methacrylate nanoparticles and Zymosan). These results seem consistent with our results,

Table IV. Complete Blood Counts in S (Saline Serum), B (Blank LNCs) and P (Paclitaxel-Loaded LNCs) Groups. Mean Values ± SD are Presented

	Haemoglobin rate (g/dL)	Leukocytes (giga/L)	Platelets count (giga/L)	Hematocrit (%)
S group (n=4)	15.1±0.1	1.6±0.5	449.5±79.2	47.6±1.2
B group (n=5)	14.5±0.8	2.2±0.5	213.0±117.5	45.8±2.7
P group (n=5)	15.2±0.3	1.5±0.3	327.0±178.4	47.4±1.2
Kruskal-Wallis test	NS	NS	NS (P=0.093)	NS

Table V. Biological Analysis in S (Saline Serum), B (Blank LNCs) and P (Paclitaxel-Loaded LNCs) Groups. Mean Values ± SD are Presented

	Natremia (mmol/L)	Kaliemia (mmol/L)	Chloremia (mmol/L)	ALAT (UI/L)	Total bilirubine level (μmol/L)	Serum creatinine (μmol/L)	C-Reactive protein (ng/mL)
S group (n=4)	151.8±3.3	24.0±2.5	101.7±1.7	25.5±4.1	0.5±0.1	11.3±1.7	16.8±6.0
B group (n=5)	153.2±3.3	24.1±2.8	102.4±2.7	29.0±5.9	1.0±0.2	9.6±1.5	23.3±4.0
P group (n=5)	153.8±4.0	19.5±3.8	104.2±1.6	37.8±11.9	0.6±0.1	13.2±1.6	22.4±8.2
Kruskal-Wallis test	NS	NS	NS	NS (P=0.136)	NS	NS	NS

which do not reveal any mark of complement activation such as haemolysis or glomerular lesions or dysfunction. Finally, biological parameters such as cell count, ions, total bilirubin, creatinine and C-Reactive protein measurements were similar in the different groups. Our study demonstrates the high level of tolerance in mice of the paclitaxel-loaded LNC dispersions at 60 mg/kg over five days. The corresponding volume of blank LNC dispersions was also well-tolerated. Toxicokinetic study is needed to confirm these first results.

Interestingly, the dose of 12 mg/kg per injection per day for 5 consecutive days for paclitaxel-loaded LNCs was more effective than Taxol® in NCI-H460 subcutaneous bearing tumours in nude mice. Ours results demonstrated an improvement of the antitumoural efficacy associated with better survival. The results could seem discordant with previous data showing that 50% growth inhibition (IC50) for Taxol® and paclitaxel-loaded LNC dispersion IC50 were 4.4 nM and 3.8 nM, respectively (42). These results are expected to be related to the intrinsic properties of the LNCs as tissue concentration driven by the EPR effect. The therapeutic effect is also amplified by sustained release of paclitaxel and P-gp inhibition by Solutol® HS15. These original data confirm the great interest of LNC dispersions in oncology field.

The single-dose toxicity study with a 28-day follow-up showed that the MTD and LD50 of paclitaxel-loaded LNCs were 96 mg/kg and 216 mg/kg, respectively. In comparison with the paclitaxel-solvent formulation Taxol®, the paclitaxel-loaded LNC dispersion tolerance in mice was increased to levels where the LD50 and MTD were, respectively, eight-fold and eleven-fold higher than the commercial formulation. The entrapment of paclitaxel in LNCs results in an improvement

Table VI. Complete Results of the Irwin Tests in Swiss Mice (a,b,c,d). Each Volume is Slowly *i.v.* Injected. Each Group Comprised of 3 Mice

Dose (mg/kg)						
12	18	24	48	96	192	288
a) Saline serum No change		No change	No change	No change	No change	Sedation + (3/3) at 15 min → 30 min
b) Taxol® No change	Convulsions ++ (1/3) at 5 min → 30 min Lethality + (1/3) at Day 10	Convulsions ++ (3/3) at 5 min → 30 min Sedation + (2/3) at 30 min → 48 h + (1/3) at 30 min → Day 4 Lethality + (2/3) at 48 h, (1/3) at Day 4				
c) High concentration, blank LNCs No change		No change	No change	No change	No change	Sedation + (3/3) at 15 min → 30 min
d) High concentration, paclitaxel-loaded LNCs No change		No change	No change	Sedation + (3/3) at 15 min → 45 min	Sedation + (3/3) at 15 min → 45 min Lethality + (1/3) at Day 13	Sedation + (3/3) at 15 min → 45 min Lethality + (3/3) at Days 4,6 and 8

of its therapeutic index in comparison with Taxol®. The blank LNC dispersion (injected at the corresponding volume of the paclitaxel-loaded LNC dispersion) was well-tolerated with no mortality or toxicity up to the level of 288 mg/kg. For this drug nanocarrier, the MTD was equal or above 288 mg/kg and the LD50 was above 288 mg/kg. The injected volume was superior to international recommendations and induced a non-physiological situation that mimics extracellular hyperhydratation. In mice with normal renal function (your experimental situation), we expected a rapid renal response based on the increase of renal excretion without any specific tissue lesion. In mice receiving saline serum, a transitory sedation has been observed at the highest dose but no renal function impairment has been observed during the 28-day follow-up. This result indicates the absence of specific toxicity due to extracellular hyperhydratation. In mice receiving high concentration, blank LNCs, no mortality was observed at the highest dosage. Our study demonstrates that an *i.v.* single dose of high concentration, blank LNC dispersion induces no mortality at the highest tested dose. This dose corresponds to a No-Observed-Adverse-Effect-Level (NOAEL). To the opposite, in mice receiving the two highest dosages of high concentration, paclitaxel-loaded LNCs, delayed lethality may be attributed to paclitaxel toxicity.

Toxicity reduction has been reported for paclitaxel solubilised with other drug-carrier systems (18–20,31,52). Two major causes can be proposed to explain this reduction in toxicity: the absence of Cremophor® EL replaced by less toxic excipients, and the modification of the biodistribution that induces less drug concentration in bone marrow. As paclitaxel-loaded LNCs are produced by solvent-free technology, such as NAB-paclitaxel, the premedication of the animals with corticosteroids and anti-histamine drugs is not required. No chemical catalyser is used during the LNC preparation, providing the assurance of an absence of residual catalyser in the final formulation. All the excipients of the LNCs are either FDA-approved or Generally Recognized as Safe (GRAS). Nevertheless, the analysis of the composition of Solutol® HS15 based on the supplier's data allowed us to identify the presence of class 2 solvents (ethylene glycol and 1,4-dioxane) and a class 3 solvent (acetic acid) below the limits provided in European Pharmacopoeia 5.4. In standard LNC formulations, Solutol® HS15 makes up 6.5% of the final weight, providing final solvent concentrations that are well below the limits given in European Pharmacopoeia 5.4.

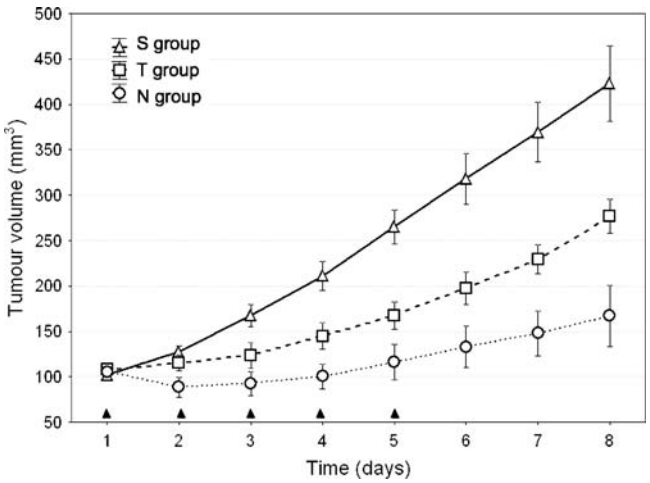

Fig. 2. Response to paclitaxel's formulations in the three groups S (saline serum), T (Taxol®) and P (paclitaxel-loaded LNCs) during the 8th first days. Mean size tumour ± SD is represented. Arrow heads indicate days of treatment.

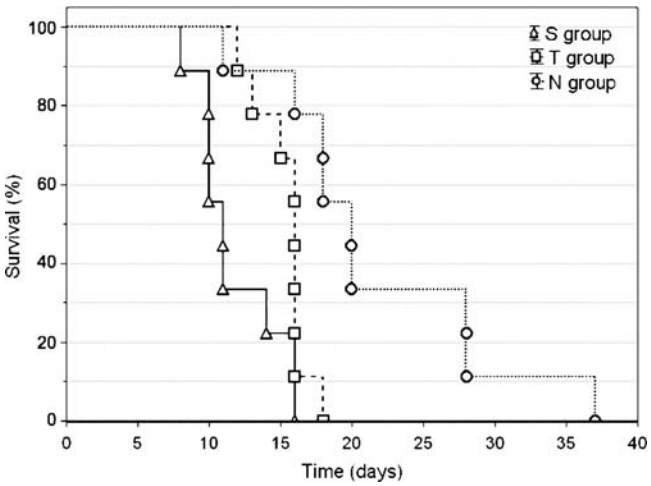

Fig. 3. Survival curves of the groups S (saline serum), T (Taxol®) and P (paclitaxel-loaded LNCs) of mice until the experimental limit point (tumour volume superior to 600 mm^3).

Our results have shown that LNC nanotechnology has not triggered any limiting toxicity effects after five consecutive days of administration at 60 mg/kg in Swiss mice. This schedule is also more effective than Taxol® in a tumour-bearing model without any premedication. These first results mean that we can set up chronic-toxicity schedule studies and complementary biological endpoints.

CONCLUSIONS

The five-day *i.v.* injection schedule of 12 mg/kg/day of paclitaxel in LNCs and blank LNCs is well-tolerated in mice and induces no toxic effects. The entrapment of paclitaxel in LNCs allows an improvement of its therapeutic index in comparison with Taxol®, based on the eight-fold and eleven-fold respective increase in the LD50 and MTD. Efficacy of paclitaxel in LNC drug nanocarrier is increased in comparison with Taxol®.

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