Safe lipid nanocapsule-based gel technology to target lymph nodes and combat mediastinal metastases from an orthotopic non-small-cell lung cancer model in SCID-CB17 mice

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Abstract

The purpose of this study is the assessment of gel technology based on a lauroyl derivative of gemcitabine encapsulated in lipid nanocapsules delivered subcutaneously or intravenously after dilution to (i) target lymph nodes, (ii) induce less systemic toxicity and (iii) combat mediastinal metastases from an orthotopic model of human, squamous, non-small-cell lung cancer Ma44-3 cells implanted in severe combined immunodeficiency mice. The gel technology mainly targeted lymph nodes as revealed by the biodistribution study. Moreover, the gel technology induced no significant myelosuppression (platelet count) in comparison with the control saline group, unlike the conventional intravenous gemcitabine hydrochloride treated group (\(P_{<0.05}\)). Besides, the gel technology, delivered subcutaneously twice a week, was able to combat locally mediastinal metastases from the orthotopic lung tumor and to significantly delay death (\(P_{<0.05}\)) as was the diluted gel technology delivered intravenously three times a week.

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Key words: Lymphatic targeting; Nanomedicine; Mediastinum; Immunomodulation

Background

Lung cancer remains the leading cause of cancer-related mortality around the world.\textsuperscript{1,2} Non-small-cell lung cancers (NSCLCs) represent 85% of all cases of lung cancers.\textsuperscript{3} Diagnosed NSCLCs are mainly present in regional and advanced extent for 22% and 56% of patients, respectively.\textsuperscript{4} At these stages, cells from a primary tumor (i.e. metastases) have migrated and have been disseminated by blood and/or lymphatic vessels to distal organs. Via the lymphatic route, metastases are trapped by lymph nodes (firstly by sentinel nodes followed by secondary and distal nodes).\textsuperscript{5} The more distal the invasion of lymph nodes is, the higher the malignancy and the poorer the prognosis is.\textsuperscript{6} Indeed, the five-year survival rate of patients with...
NSCLC treated by current treatment methods is estimated at 42% for a stage of N0 (without regional lymph node metastases) to 7% for N3 corresponding to the latest stage of lymph node implication. Patients with mediastinum lymph node metastases (N2 and N3) are considered in the advanced stage III and are mainly treated by a combination of radiotherapy and chemotherapy with poor improvement in long-term survival and at the expense of a large rise in Grade 3 toxicities. Moreover, mediastinal lymph node metastases can induce the “superior vena cava (SVC) syndrome”, which is an array of clinical signs and symptoms due to the impairment of blood flow through the SVC. The flow impairment is usually caused by the enlargement of mediastinal lymph nodes that compress the SVC. The symptoms include dyspnea, coughing, and swelling of the face, neck, upper trunk, and extremities. Furthermore, headache, confusion, and possibly coma can result from cerebral edema. The SVC syndrome from lung malignancies is treated by chemotherapy and/or radiotherapy or by endovascular stenting in emergency cases.

Conventional chemotherapy is mainly delivered by perfusion but includes major limitations: high systemic toxicity and poor lymph node exposure and retention. In consequence, an efficient drug delivery system increasing lymph node exposure and retention while decreasing systemic toxicity could be useful against lymph-metastatic cancers. With this aim, a nanocarrier system, lipid nanocapsules (LNCs), loaded with the lipophilic pro-drug gemcitabine (Gem-C12) has been developed. Gemcitabine is a third generation agent composed of the platinum based doublet chemotherapy used as first-line treatment for advanced NSCLC patients with good performance status. However, due to its hydrophilic nature and its low membrane permeability and also due to its extensive deamination in plasma and tissues by cytidine deaminase, its plasma elimination half-life (t1/2) after 30 min perfusion is extremely short (42-94 min). Therefore, a high dose regimen is required which mainly leads to dose-limiting myelosuppression in approximately two-thirds of patients. Moreover, different resistance mechanisms against gemcitabine have evolved in cancer cells; this fostered the emergence of chemically-modified gemcitabine. The Gem-C12 (i.e. 4-N-lauroyl-gemcitabine) was synthesized by Tokunaga et al to decrease the deamination in blood responsible for inactive metabolite formation and to decrease the hydrophilicity responsible for poor drug loading in lipophilic nanosystems.

The LNCs loaded with Gem-C12 have previously demonstrated the ability (i) to form a hydrogel when Gem-C12 is encapsulated in LNCs, (ii) to release Gem-C12-based LNCs at a rate depending on the Gem-C12 concentration, (iii) to be in vitro injected through 18 or 21G needle, (iv) to form a suspension after dilution, and (v) to exert a higher-than-average antitumor efficacy in a highly aggressive patient-like lymphogenous metastatic model of human NSCLC. This lymphogenous metastatic model mimics the spreading of metastases in mediastinum from the primary tumor implanted in the lung of severe combined immunodeficiency mice with a CB17 genetic background (SCID-CB17) as observed for NSCLC patients.

Methods

Material

Labrafac® WL 1349 (caprylic–capric acid triglycerides) was provided by Gattefosse S.A. (Saint-Priest, France). Kolliphor® HS15 (mixture of free polyethylene glycol 660 and polyethylene glycol 660 hydroxystearate) was supplied by BASF (Ludwigshafen, Germany). Sodium chloride, acetone and ethanol were purchased from VWR (Fontenay-sous-Bois, France). Span® 80 (sorbitan monoooleate), Tween® 80 (polysorbate 80) and 5-aminolevulinic acid (ALA) were purchased from Sigma (St Quentin Fallavier, France). 1,1′-Dioctadecyl-3,3′,3′-tetramethylindodicarbocyanine (DiD) was provided by Life Technologies (Saint-Aubin, France). Methanol was analytical grade purchased from Fischer Scientific (Loughborough, United Kingdom). Water was obtained from a MilliQ filtration system (Millipore, Paris, France). 4-(N)-lauroyl-gemcitabine (Gem-C12) was synthesized and characterized as described elsewhere.

Preparation of the formulations

The LNC-based gel technology was prepared following the phase-inversion temperature (PIT) process. Briefly, Gem-C12 (0.062 g) was first solubilized (5%, ratio Gem-C12/Labrafac® w/v) in a mix of Labrafac® WL 1349 (1.24 g), Span® 80 (0.25 g) and acetone that was evaporated before the addition of Kolliphor® HS15 (0.967 g), NaCl (0.045 g) and water (1.02 g). All were then mixed and heated to 75 °C under magnetic stirring at 500 rpm followed by cooling to 45 °C (rate of 5 °C/min). Three cycles were performed and at the last cooling phase, a sudden dilution with 2.12 g room-temperature water was carried out at 60 °C. LNCs loaded with Gem-C12 spontaneously formed a hydrogel with a waxy aspect. The gelation process was considered to be achieved after 24 h at 4 °C. LNCs loaded with the prodrug (Gem-C12 LNC) and used in a liquid form were acquired by the 4-fold dilution of the formulation directly after the process, before the establishment of the hydrogel. Non-loaded LNCs were prepared in the same way as Gem-C12 LNCs hydrogel but without Gem-C12 and acetone. Fluorescent LNCs were obtained by adding the fluorescent probe (DiD) with the other reagents at a final concentration of 0.1% of Labrafac® (w/v).

Free Gem-C12 was dissolved in ethanol, Tween® 80 and water (87.6/5.5/6.9 v/v/v) as a micellar system for in vivo experiments. The
commercial gemcitabine hydrochloride was used. Gemcitabine Hospira 38 mg/mL, diluted at the required concentration with 0.9% NaCl.

Characterization of LNCs-based formulations

The hydrodynamic diameter (Z-average), polydispersity index (Pdi) and zeta potential of LNCs-based formulations were determined by dynamic light scattering and laser doppler electrophoresis using a Zetasizer® Nano series DTS 1060 (Malvern Instruments S.A., Worcestershire, UK). LNC-based formulations were diluted 1:60 (v/v) in deionized water in order to ensure a convenient scatter intensity on the detector; each measurement was done in triplicate at 25 °C. The drug loading was determined after dialysis, using an ultra performance liquid chromatography apparatus with UV spectroscopy detection as previously described; each measurement was done in triplicate.

Animals

For pharmacokinetic and biodistribution studies, female nude Swiss mice were purchased from Harlan (Gannat, France) and for in vivo efficacy and tolerance evaluations, male SCID-CB17 mice were purchased from Charles River (L’Arbresle, France). The animals were housed and maintained at the university animal facility (SCAHU) in ventilated plastic cages with hardwood chips bedding in an air-conditioned room with a 12-hour-light/12-hour-dark cycle. All the animal experiments were carried out in accordance with EU Directive 2010/63/EU and with the agreement of the “Comité d’Ethique pour l’Expérimentation Animale des Pays de la Loire” (authorization CEEA; 2012–37 and 2012–73).

Pharmacokinetic and biodistribution of fluorescent LNC-based formulations in healthy nude mice

Mice (9 weeks old, n = 5 for each group) were anesthetized (isoflurane) and either 110 μL of DiD-GemC12 LNCs was injected subcutaneously (sc) behind the neck, or 110 μL of DiD LNCs was injected intravenously (iv) into the tail vein with the aim of delivering the same amount of DiD-loaded LNCs. After various lengths of time, from 1 h to 336 h, the mice were sacrificed and the blood was removed by cardiac puncture in a venous blood collection tube containing Li-heparin (Tube Micro from SARSTEDT, Marnay, France). The organs were then removed for the biodistribution study. Plasma from each blood sample was obtained after 10 min of centrifugation at 2000 g.

The DiD concentrations in plasma (encapsulated inside LNCs) over time were determined using a microplate reader Fluoroscan Ascent® (Labsystems SA, Cergy-Pontoise, France) at excitation and emission wavelengths of 646 and 678 nm, respectively. The DiD concentrations in plasma were intrapolated from the linear curve between 0.006 and 12 μg/mL (r² > 0.999). The recovered DiD concentrations were then normalized in function of the animal’s weight, assuming that blood represents 7.5% of mouse body weight. Pharmacokinetic data were determined by iv bolus and extravascular non-compartmental analysis (for iv and sc administration) respectively of the recovered systemic DiD concentration over time using Kinetica 4.1.1 software (Thermo Fisher Scientific, Villebon sur Yvette, France). The trapezoidal calculation method was used to determine the area under the curve (AUC) during the whole experimental period (from 1 to 336 h) without extrapolation. The t1/2 was calculated from 1 to 8 h for t1/2 distribution and from 8 to 336 h for t1/2 elimination.

For the biodistribution study, the organs (i.e., kidneys, liver, spleen, lung, heart, stomach, and intestine) and lymph nodes (i.e., inguinal, axillary, cervical and brachial lymph nodes) were removed and analyzed by a fluorescence CRI Maestro™ imaging system (Woburn, USA). Semi-quantitative data were obtained by setting a time exposure of 10 ms between 630 and 800 nm, unmixing the generated cube, extracting the background, and drawing the regions of interest from fluorescence images (Figure 1, A). The software Maestro 2.10 (Woburn, USA) was used to calculate the average signal expressed in photon/cm²/s.

Cell culture and inoculation preparation for lung implantation

An Ma44-3 cell line derived from the human squamous NSCLC carcinoma cancer cell line Ma44 by limit dilution method was kindly provided by Prof. Kondo (Department of Oncological and Regenerative Surgery, School of Medicine, University of Tokushima, Japan) and was cultured in RPMI 1640 (Lonza, Verviers, Belgium) supplemented with 10% heat-inactivated fetal bovine serum (Lonza, Verviers, Belgium), 100 U/mL of penicillin, 100 μg/mL of streptomycin and 0.250 μg/mL of amphotericin B from Sigma (St Quentin Fallavier, France) at 37 °C in a humidified incubator with 5% CO₂. For lung implantation, the cells were harvested at 70-80% confluence using trypsin-EDTA (Lonza, Verviers, Belgium) that was inactivated by the culture medium. Then, the cell suspension was centrifuged at 144 g for 5 min and the supernatant was removed and replaced by RPMI 1640 containing 0.1% bovine serum albumin fraction V pH 7.0 (PAÄ GmbH, Pasching, Austria) which was then mixed with Matrigel® (DB Biosciences, Bedford, MA) to obtain an inoculum of 2.0 × 10⁶ tumor cells/mL with 400 μg/mL of Matrigel® (BD sciences, Bedford, USA). The inoculum was kept on ice for a maximum of 2 h.

Orthotopic intrapulmonary implantation procedure

The orthotopic intrapulmonary implantation procedure was performed as previously reported. Briefly, the 6-week-old SCID-CB17 mice were maintained in the right lateral decubitus and anesthetized by isoflurane inhalation. A 1 cm transverse incision was made on the left lateral skin just below the inferior border of the scapula of the SCID-CB17 mice. The muscles were separated from the ribs by sharp dissection, leaving the intercostal muscles visible. 10 μL of cell suspension (2.0 × 10⁶ cells) was inoculated with a 30-gauge needle to a depth of about 3–5 mm into the lung through the intercostal muscles and after, the needle was promptly pulled out. Mice were maintained in the right lateral decubitus position after injection and the skin incision was closed with 3–0 silk (Ethicon, St-Stevens-Woluwe, Belgium). The mice were observed until complete recovery.

In vivo efficacy on the lymphogenous metastatic model

The in vivo efficacy of different treatments on survival was evaluated on a lymphogenous metastatic model. Seven groups of 10 SCID-CB17 mice implanted with 2.0 × 10⁶ Ma44-3 cells in the left lung were randomly established. Five groups were treated by iv (145 μL at each injection by the tail vein) on days 5, 7 and 9 after tumor grafting, either with (i) NaCl 0.9% solution (saline iv), (ii) non-loaded LNCs (non-loaded LNCs iv), (iii) commercial
gemcitabine hydrochloride (gemcitabine iv), (iv) Gem-C12 micelles (Gem-C12 iv) or (v) liquid form of Gem-C12-loaded LNCs (Gem-C12 LNCs iv). Two groups were treated by sc route (55 μL at each injection behind the neck) on days 5 and 9 after tumor grafting either with (i) non-loaded LNCs (non-loaded LNCs sc) or (ii) gel form of Gem-C12-loaded LNCs (Gem-C12 LNCs sc). The total LNCs delivered dose of non-loaded LNCs was the same as the Gem-C12-loaded LNCs for iv or sc. In
groups treated by gemcitabine hydrochloride or Gem-C12 (in LNCs or not), the total dose delivered to mice was 40 mg (molar equivalent gemcitabine hydrochloride) per kilogram of body weight. The mice were observed every day and body weight measurements were performed daily during the treatment period and then three times a week. By applying the criteria for euthanasia of experimental animals, the mice were sacrificed and then three times a week. By applying the criteria for measurements were performed daily during the treatment period weight. The mice were observed every day and body weight.

Visualization of the fluorescent LNC-based formulations in lung and mediastinum in tumor bearing mice

Visualization of the lung and mediastinal distribution of fluorescent LNC-based formulations delivered sc or iv after dilution was performed after 4 h on tumor-grafted SCID-CB17 mice. On day 5 post tumor grafting, a group of 3 mice received an iv injection of DiD LNCs and the other group of 3 mice received an sc injection of DiD-Gem-C12 LNCs to deliver the same amount of DiD LNCs. Moreover, both groups received orally 400 mg/kg of ALA in PBS (200 μL) to detect tumor mass. Four hours after the respective administrations, the mice were killed and dissected for imaging the lung and the mediastinum area. Fluorescence imaging was carried out using the CRI Maestro system between 500 and 635 nm (for Pp IX for tumor mass visualization) and 630 and 800 nm (for DiD for LNC visualization) using automatic exposure.

In vivo tolerance evaluation of the formulations

Mice (7 weeks old, 5 per group) received 145 μL of an iv injection of saline (saline iv), gemcitabine hydrochloride (gemcitabine iv), Gem-C12 micelles (Gem-C12 iv), non-loaded LNCs (non-loaded LNCs iv) or liquid form of Gem-C12-loaded LNCs (Gem-C12 LNCs iv) dispersions three times a week for one week (Monday, Wednesday and Friday). Ten mice (5 per group) received 55 μL of an sc injection of non-loaded LNCs (non-loaded LNCs sc) or gel form of Gem-C12-loaded LNCs (Gem-C12 LNCs sc) twice a week for one week (Monday and Friday). The LNC delivered dose of non-loaded LNCs was the same as Gem-C12-loaded LNCs for iv or sc administration. In groups treated by gemcitabine hydrochloride or Gem-C12 (in LNCs or in micelles), the dose delivered to mice was 40 mg (molar equivalent gemcitabine hydrochloride) per kilogram of body weight. Hematology and biochemical assays on blood were carried out for each mouse 8 days after the first injection, to assess the tolerance to treatment. Blood sampling was performed by cardiac puncture in anesthetized mice; half of the blood sample was placed in a venous blood collection ethylene-diamine-tetraacetic acid tube (K2E tube from BD Microtainer, NJ, USA) for hematology studies and the other half in a venous blood collection tube containing heparin lithium (Tube Micro from SARSTEDT, Marnay, France) which were then centrifuged at 10,000 g for 10 min to remove the plasma and evaluate the blood biochemical markers. The hematological parameters were determined in the Hematology Ward of the Academic Hospital of Angers with an XE-2100 hematology analyzer (Sysmex). Plasma biochemistry analyses were carried out at the Biochemistry Ward of the Academic Hospital of Angers on an Architect C16000 (Abbott). Statistical comparisons of data were analyzed by a non-parametric one-way analysis of variance (Kruskal–Wallis test), followed by Dunn’s post-hoc test for pairwise comparisons.

Results

LNC-based formulations

The physicochemical characteristics of LNC-based formulations are presented in Table 1. With the addition of Gem-C12 into LNCs, a hydrogel was formed that can be injected using a syringe. Once the gel was diluted 4-fold in water, a liquid LNCs suspension was obtained (Gem-C12 LNCs iv). LNCs formulations prepared by the FIT method displayed a Z-average (i.e., hydrodynamic diameter) between 53 and 68 nm, depending on the presence of the amphiphilic Gem-C12 and/or DiD in the LNCs surface, and a polydispersity index in all cases less than 0.1, which means that monomodal and monodispersed distributions were obtained. In all cases, the zeta potential was slightly negative, from −5 to −10 mV. Similarly to many hydrophobic drugs, Gem-C12 was well-solubilized in the LNCs, forming a mono- or gel form of Gem-C12-loaded LNCs (Gem-C12 LNCs sc) or liquid form of Gem-C12-loaded LNCs (Gem-C12 LNCs iv) dispersions three times a week for one week (Monday and Friday). The LNC delivered dose of non-loaded LNCs was the same as Gem-C12-loaded LNCs for iv or sc administration. In groups treated by gemcitabine hydrochloride or Gem-C12 (in LNCs or in micelles), the dose delivered to mice was 40 mg (molar equivalent gemcitabine hydrochloride) per kilogram of body weight. Hematology and biochemical assays on blood were carried out for each mouse 8 days after the first injection, to assess the tolerance to treatment. Blood sampling was performed by cardiac puncture in anesthetized mice; half of the blood sample was placed in a venous blood collection ethylene-diamine-tetraacetic acid tube (K2E tube from BD Microtainer, NJ, USA) for hematology studies and the other half in a venous blood collection tube containing heparin lithium (Tube Micro from SARSTEDT, Marnay, France) which were then centrifuged at 10,000 g for 10 min to remove the plasma and evaluate the blood biochemical markers. The hematological parameters were determined in the Hematology Ward of the Academic Hospital of Angers with an XE-2100 hematology analyzer (Sysmex). Plasma biochemistry analyses were carried out at the Biochemistry Ward of the Academic Hospital of Angers on an Architect C16000 (Abbott). Statistical comparisons of data were analyzed by a non-parametric one-way analysis of variance (Kruskal–Wallis test), followed by Dunn’s post-hoc test for pairwise comparisons.

<table>
<thead>
<tr>
<th>Z-Ave&lt;sup&gt;a&lt;/sup&gt; (nm)</th>
<th>Pdi&lt;sup&gt;b&lt;/sup&gt;</th>
<th>ζ-Pot&lt;sup&gt;c&lt;/sup&gt; (mV)</th>
<th>Gem-C12 payload (mg/mL)</th>
<th>Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-loaded LNCs (iv)</td>
<td>68 ± 3</td>
<td>0.08 ± 0.02</td>
<td>−8 ± 3</td>
<td>Liquid</td>
</tr>
<tr>
<td>Non-loaded LNCs (sc)</td>
<td>67 ± 2</td>
<td>0.07 ± 0.01</td>
<td>−10 ± 5</td>
<td>Liquid</td>
</tr>
<tr>
<td>Gem-C12 LNCs (sc)</td>
<td>53 ± 2</td>
<td>0.07 ± 0.01</td>
<td>−7 ± 4</td>
<td>Hydrogel</td>
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<tr>
<td>Gem-C12 LNCs (iv)</td>
<td>53 ± 1</td>
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<td>−7 ± 3</td>
<td>2.75 ± 0.05 Liquid</td>
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<td>DiD-Gem-C12 LNCs (sc)</td>
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<td>0.04 ± 0.02</td>
<td>−5 ± 2</td>
<td>11 ± 0.2 Hydrogel</td>
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<tr>
<td>DiD LNCs (iv)</td>
<td>55 ± 2</td>
<td>0.06 ± 0.02</td>
<td>−7 ± 4</td>
<td>Liquid</td>
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n = 3; mean ± SD.

<sup>a</sup> Z-Average.
<sup>b</sup> Polydispersity index.
<sup>c</sup> Zeta potential.

Biodistribution and pharmacokinetic of LNC-based formulations

LNCs loaded with the fluorescent dye DiD were used to evaluate their tissue biodistribution and pharmacokinetics after iv (suspension of DiD LNCs) and sc (hydrogel of DiD-Gem-C12 LNCs) administrations in nude Swiss mice. The fluorescent dye DiD was chosen to be encapsulated in the LNCs because (i) it is a near-infrared fluorophore limiting the auto-fluorescence wavelength emitted by animals, and (ii) it is weakly fluorescent in aqueous media, but highly fluorescent in a lipophilic vehicle, and (iii) there is no dye release (strong fluorescence labeling stability of the nanocarrier).

The semi-quantitative fluorescence of the organs extracted after 1, 4, 8, 48, 96, and 336 h is reported in Figure 1, C-F and Figure S1 in supplementary information. DiD LNCs iv and
DiD-Gem-C12 LNCs were rapidly distributed in the lymph nodes of the nude mice. An immediate accumulation in the liver and spleen after 4 and 8 h was observed with DiD LNCs iv. After 48 h, a decrease of the accumulation was observed for these organs to totally disappear after 336 h (Figure 1, C). DiD-Gem-C12 LNCs accumulated exclusively in the lymph nodes close to the injection site (i.e., in axillary, cervical and brachial lymph nodes) in similar amounts to the first hours (i.e., 1, 4 and 8 h) as DiD LNCs iv but was much higher after a few days (i.e., 48, 96 and 336 h) (Figure 1, F). Moreover, DiD-Gem-C12 LNCs presented a much lower plasmatic exposition than DiD LNCs iv (Figure 1, B) resulting in a lower AUC of 4 μg · h/mL in comparison with an AUC of 174 μg · h/mL, respectively. Besides, DiD-LNCs was more rapidly cleared from the systemic circulation after iv administration with a t1/2 elimination of 19 h in comparison to 32 h found for the sc injection of the hydrogel. Indeed, the latter presented controlled-release properties.

In vivo efficacy of the LNC-based formulations

The patient-like lymphogenous metastatic Ma44-3 preclinical model was used to evaluate the efficacy of LNC-based gel technology. Five days post-tumor graft in a left lung lobe, when micrometastatic foci were detected in mediastinum (Figure S2-A), a comparative efficacy study on randomized groups was performed with different treatments and schedules of administration. The dose of 40 mg (molar equivalent dose of gemcitabine hydrochloride) per kilogram of body weight delivered twice or three times a week by the iv (i.e., Gem-C12 sc) or iv (i.e., Gem-C12 iv) route or Gem-C12 LNCs (liquid form) for sc route, respectively. Higher doses or similar doses with fewer injections caused drastic weight loss or the death of mice (data not shown). The survival times of similar experimental animals were plotted on the Kaplan–Meier curves as shown in Figure 2, A. Mice of the saline iv and non-loaded LNCs iv or sc groups were characterized by a very short lifespan after tumor implantation without significant difference (P > 0.05, log-rank test) (Table S1 in supplementary material). In the treated groups by gemcitabine hydrochloride or Gem-C12, all groups except gemcitabine iv (i.e., Gem-C12 iv, Gem-C12 LNCs iv and Gem-C12 LNCs sc) showed significant differences (P < 0.05, log-rank test) in comparison with the saline iv group (Table S1 in supplementary material). However, Gem-C12 LNCs sc showed a significantly prolonged lifespan (P < 0.05, log-rank test) in comparison to its control group (i.e., non-loaded LNCs sc); and Gem-C12 LNCs iv showed a significantly prolonged lifespan (P < 0.05, log-rank test) in comparison to the gemcitabine iv group (Table S1 in supplementary material). The weight evolution of the different mice groups remained similar during the experiment with a short stabilization during the treatment period (Figure 2, B).

Visualization of fluorescent LNC-based formulations in lung and mediastinal lymph nodes

The in vivo behavior of LNC-based formulations was then assessed following the iv injection of DiD-LNCs and the sc injection of DiD-Gem-C12 in tumor-bearing SCID-CB17 mice to better understand the result of antitumor efficacy. The fluorescence images obtained 4 h after DiD-loaded LNCs formulation injections are presented in Figure 3. DiD LNCs is visible in the entire lung of the three mice after iv injection (Figure 3, B) because at this time, the LNCs mainly remained in the blood as confirmed by plasmatic exposition (Figure 1, B) and because the lung is a highly vascularized organ. However, no accumulation of DiD LNCs (after iv administration) was seen after 4 h in mediastinal lymph nodes contrary to the other lymph nodes during the biodistribution study (Figure 1, E). For DiD-Gem-C12 LNCs administered by sc route, only an intense local accumulation in mediastinal lymph nodes was observed (Figure 3, D) and not in the entire lung of the three mice. Mediastinal lymph nodes are known to be close to the thymus.30 Indeed, a negligible plasma exposition for DiD-Gem-C12 LNCs was previously seen in Figure 1, B.

Figure 2. (A) Kaplan–Meier survival and (B) weight evaluation curves of 10 SCID-CB17 mice grafted with 2 × 10⁴ Ma44-3 cells (implanted in the left lobe) and randomized on day 0. The negative controls were iv saline treated group (*), iv non-loaded LNC treated group (■) and sc non-loaded LNC treated group (○). The groups were treated by gemcitabine hydrochloride (▲) or Gem-C12 loaded in micelles (●) or loaded in LNC (◆), delivered by iv route; or Gem-C12 loaded in LNC (◇), delivered by sc route. The dose was 40 mg (molar equivalent gemcitabine hydrochloride) per kilogram of body weight beginning on day 5 post-tumor graft. The treatments were administered iv (continuous line) via the tail vein three times a week or by sc (dashed line) twice a week.
Tolerance of the LNC-based gel technology on SCID-CB17 mice

To evaluate if the different treatments used in SCID-CB17 mice induced myelosuppression, the complete blood count was performed 8 days after the first injection as in clinic and are presented in Table 2. A significant decrease of platelet count was only observed with the group treated by gemcitabine hydrochloride iv in comparison to the saline iv control group (*Pb0.05, Kruskal–Wallis test) and no difference was measured for Gem-C12 non or loaded in LNCs and delivered iv or sc in the respective schedule. SCID-CB17 mice are severe combined immunodeficiency mice characterized by severe lymphopenia, which prevents the analysis of the complete granulocyte count. Plasma biochemical parameters such as aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), alkaline phosphatase (PH ALK) and serum creatinine were evaluated (Table 3). No significant difference was seen between the different groups in comparison to the control saline iv group, except for a significant decrease of PH ALK for Gem-C12 not loaded in LNCs, but formulated with ethanol as a co-solvent and in surfactant micelles (*Pb0.05, Kruskal–Wallis test).

Discussion

Drug delivery to the lymphatic system is a good option to combat metastasis spreading from a primary tumor via the lymphatic system and to modulate immunity. Indeed, the lymphatic system filters particles (e.g., nanomedicine) or cells (e.g., cancer cells) from interstitial fluid and supports the activity of the lymphocytes, which furnish immunity, or resistance, to the specific disease-causing agents. In this study, using the sc administration route, slightly negative-charged 50 nm LNCs (polyethene glycol 660-based surface) composing the hydrogel were chosen because they could be the most appropriate method for lymphatic drainage and lymph node retention. The gel form of DiD-Gem-C12 LNCs progressively released 50 nm LNCs in the interstitial fluid. Released LNCs passed rapidly into the lymphatic capillary vessels and were drained until lymph nodes as observed by the specific accumulation in adjacent lymph nodes: axillary, brachial, cervical (Figure 1, F) and mediastinum lymph nodes (Figure 3, B). When DiD-LNCs were delivered intravenously, the LNCs were taken up by the mononuclear phagocytic system that recognized the adsorption of complement proteins on the LNCs surface, as observed by their rapid accumulation in the liver and spleen (Figure 1, C), and as previously reported by Hirsjarvi et al. DiD-LNCs were also partly carried up to lymph nodes, as revealed by their accumulation in all the studied lymph nodes (Figure 1, E). However, they were not accumulated in mediastinum lymph nodes, where the interstitial fluid of the lung is drained. Indeed, healthy blood vessels of the lung have poor permeability due to small pores in postcapillary venules (<6 nm). As a consequence, nanomedicine such as 50 nm-LNCs were poorly extravasated in lung parenchyma and then poorly transported.

Table 2

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<th>WBC (giga/L)</th>
<th>RBC (tera/L)</th>
<th>HGB (g/dL)</th>
<th>HCT (%)</th>
<th>PLT (giga/L)</th>
</tr>
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<tr>
<td>Saline iv</td>
<td>0.2 ± 0.1</td>
<td>9.8 ± 0.3</td>
<td>15.1 ± 0.3</td>
<td>47 ± 1</td>
<td>469 ± 50</td>
</tr>
<tr>
<td>Gemcitabine iv</td>
<td>0.2 ± 0.1</td>
<td>9.2 ± 0.3</td>
<td>14.0 ± 0.4</td>
<td>45 ± 1</td>
<td>245 ± 63*</td>
</tr>
<tr>
<td>Gem-C12 iv</td>
<td>0.3 ± 0.2</td>
<td>9.1 ± 0.6</td>
<td>14 ± 1</td>
<td>45 ± 2</td>
<td>275 ± 63</td>
</tr>
<tr>
<td>Non-loaded LNC iv</td>
<td>0.6 ± 0.3</td>
<td>9.8 ± 0.2</td>
<td>15.1 ± 0.3</td>
<td>47.7 ± 0.9</td>
<td>465 ± 44</td>
</tr>
<tr>
<td>Gem-C12 LNC iv</td>
<td>0.14 ± 0.03</td>
<td>8.9 ± 0.2</td>
<td>13.7 ± 0.3</td>
<td>43.1 ± 0.9</td>
<td>284 ± 24</td>
</tr>
<tr>
<td>Non-loaded LNC sc</td>
<td>0.4 ± 0.1</td>
<td>9.4 ± 0.7</td>
<td>14 ± 1</td>
<td>46 ± 3</td>
<td>453 ± 20</td>
</tr>
<tr>
<td>Gem-C12 LNC sc</td>
<td>0.26 ± 0.06</td>
<td>9.4 ± 0.5</td>
<td>14.5 ± 0.8</td>
<td>45 ± 2</td>
<td>281 ± 83</td>
</tr>
</tbody>
</table>

n = 5; mean ± SD. WBC: complete granulocyte count. RBC: red blood cell count. HGB: hemoglobin rate. HCT: hematocrit. PLT: platelet count. *P < 0.05.

Figure 3. Visualization of the lungs and mediastina of three SCID-CB17 mice on day 5 after tumor implantation, 4 hours after oral administration of 400 mg/kg of ALA and either 4 h after iv administration of DiD LNCs using (A) Protoporphyrin IX (Pp IX) or (B) DiD visualization mode; or sc administration of DiD-Gem-C12 LNCs using (C) Pp IX or (D) DiD visualization mode using a CRI Maestro system. Pp IX mode revealed the lung (green-brown color), the lung tumor (slight red color), the mediastinum (green color) and the thymus (red color). The DiD mode revealed LNCs distribution in the lung and the mediastinum. Arrows and circles in the picture indicate the localization of detectable, mediastinal lymph nodes and tumor mass, respectively.
myelosuppression, contrary to conventional systemic gemcitabine hydrochloride.

References


