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RESEARCH PAPER

Treatment of 9L Gliosarcoma in Rats by Ferrociphenol-Loaded Lipid Nanocapsules Based on a Passive Targeting Strategy via the EPR Effect

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ABSTRACT

Purpose To study a passive targeting strategy, via the enhanced permeability and retention effect following systemic administration of lipid nanocapsules (LNCs) loaded with ferrociphenol, FcdiOH.

Methods Long chains of polyethylene glycol (DSPE-mPEG2000) were incorporated onto the surface of LNCs by post-insertion technique. Stealth properties of LNCs were investigated by *in vitro* complement consumption and macrophage uptake, and *in vivo* pharmacokinetics in healthy rats. Antitumour effect of FcdiOH-loaded LNCs was evaluated in subcutaneous and intracranial 9L gliosarcoma rat models.

Results LNCs and DSPE-mPEG2000-LNCs presented low complement activation and weak macrophage uptake. DSPE-mPEG2000-LNCs exhibited prolonged half-life and extended area under the curve in healthy rats. In a subcutaneous gliosarcoma model, a single intravenous injection of FcdiOH-LNCs (400 μL, 2.4 mg/rat) considerably inhibited tumour growth when compared to the control. DSPE-mPEG2000-FcdiOH-LNCs exhibited a strong antitumour effect by nearly eradicating the tumour by the end of the study. In intracranial gliosarcoma model, treatment with DSPE-mPEG2000-FcdiOH-LNCs and FcdiOH-LNCs statistically improved median survival time (28 and 27.5 days, respectively) compared to the control (25 days).

Conclusion These results demonstrate the interesting perspectives for the systemic treatment of glioma thanks to bio-organometallic chemotherapy via lipid nanocapsules.

KEY WORDS ectopic · FcdiOH · orthotopic · PEGylated nanoparticles · stealth properties

INTRODUCTION

From the success of cisplatin in cancer therapy about 40 years ago, research in metal-based drugs for cancer therapy has entered a fascinating field, not only by synthesising new effective moieties, but also by designing novel systems for the purpose of effective and specific drug delivery to targeted tissues. For instance, Jaouen and co-workers have recently developed a new series of organometallic tamoxifen derivatives by adding a potentially cytotoxic ferrocene moiety to the tamoxifen skeleton (1–3). Among these synthesised derivatives, the compound of 2-ferrocenyl-1,1-bis(4-hydroxyphenyl)-but-1-ene, a so-called ferrociphenol compound (FcdiOH), has proved to be an effective cytostatic compound with an IC50 of about 0.5 μM on 9L gliosarcoma cells, and highly-reduced toxicity was observed on astrocytes (IC50 = 50 μM) (4). This suggests that FcdiOH is toxic to brain tumour cells, which present a high cell division potential, but are harmless towards healthy cells. However, the pharmacological application of this molecule can lead to poor bioavailability owing to its hydrophobic properties. Therefore, it should be interesting to load this

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compound into nanocarriers to facilitate their *in vivo* administration (5).

Our laboratory has recently developed and patented a nanoscale system, so-called lipid nanocapsules (LNCs), that are characterised by a hybrid structure between polymer nanocapsules and liposomes (6). The LNC formulation is based on the phase-inversion phenomenon of a micro-emulsion leading to the formation of stable LNCs with low polydispersity (6). Owing to their oily core, such nanocarriers demonstrate a high loading capacity for various lipophilic drugs (7). Recently, Allard *et al.* demonstrated that FcdiOH was well encapsulated into the oily core of LNCs (FcdiOH-LNCs) at high loading levels (6.5 mg FcdiOH per gram of LNC suspension, corresponding to 2% w/w dry weight) with a high FcdiOH encapsulation efficiency (>98%) (4). Moreover, the *in vitro* cytostatic activity of FcdiOH was totally conserved after encapsulation. With respect to its *in vivo* antitumour activity, FcdiOH-LNC treatment resulted in remarkable effects in an ectopic gliosarcoma rat model, following an intratumoural injection, as well as in an orthotopic gliosarcoma rat model by means of the convection-enhanced delivery (CED) technique (4,8).

Intravenous injection is considered an appropriated route of administration for chemotherapy in cancer patients. It is easier to handle than alternatives and removes the burden of surgical operation for cancer patients. Moreover, following an intravenous injection, the drug will be dispersed in the body thanks to blood circulation, allowing the anticancer agent/drug-loaded nanocarriers to reach the dissiminated tissues, *i.e.* metastatic tumours. However, the systemic administration of conventional nanoparticles usually leads to their rapid elimination from the blood circulation due to opsonisation, followed by their regconition and elimination from circulation by the mononuclear phagocyte system (MPS) (9,10). This phenomenon has also been observed in the case of conventional LNCs. Indeed, despite their shell consisting of polyethylene glycol (PEG 660) at high density, they were rapidly eliminated from the blood circulation system (around 21–22 min. in rats) due to the short length of the PEG chains (11). As a consequence, attempts to prolong the circulation time of LNCs led to the surface modification by coating them with longer PEG chains (9). Indeed, long chains of PEG are able to create a zone of steric hindrance around the carriers owing to the hydrophilicity and flexibility of PEG. This prevents nanoparticles from being detected and removed by the MPS cells and, hence, prolongs their biological half-life. Thanks to this long-circulating property, PEGylated nanoparticles can accumulate passively in the tumours after an intravenous injection through the enhanced permeability and retention (EPR) effect (12). Recently, LNCs coated with 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol)2000] (DSPE-mPEG2000) showed a prolonged circulation time in healthy mice and enhanced accumulation in subcutaneous U87MG gliomas after an intravenous injection into nude mice (13). As far as brain tumours are concerned, due to the specific disruption of the blood-brain barrier at the tumour site (14), the effective delivery of a drug via systemic injection has already been achieved with differing results (15–18).

The present study investigated the stealth properties of FcdiOH-LNCs *versus* DSPE-mPEG2000-FcdiOH-LNCs in terms of *in vitro* macrophage uptake and complement activation. Their corresponding blood kinetic profiles after an intravenous injection into rats were also assessed. Finally, the anticancer effect of these carriers after a single intravenous injection was evaluated in an ectopic and an orthotopic gliosarcoma model in rats (subcutaneously and intracranially implanted 9L gliosarcoma, respectively).

MATERIALS AND METHODS

Materials

Ferrociphenol compound (2-ferrocenyl-1,1-bis(4-hydroxyphenyl)-but-1-ene) named FcdiOH was prepared by a McMurry coupling reaction (19). The lipophilic Labrafac® CC (caprylic-capric acid triglycerides) was kindly provided by Gattefosse S.A. (Saint-Priest, France). Lipoïd® S75-3 (soybean lecithin at 69% of phosphatidylcholine) and Solutol® HS15 (a mixture of free polyethylene glycol 660 and polyethylene glycol 660 hydroxystearate) were gifts from Lipoïd Gmbh (Ludwigshafen, Germany) and BASF (Ludwigshafen, Germany), respectively. NaCl was obtained from Prolabo (Fontenay-sous-bois, France). Deionised water was acquired from a Milli-Q plus system (Millipore, Paris, France) and sterile water from Cooper (Melun, France). 1,2-DiStearoyl-sn-glycero-3-PhosphoEthanolamine-N-[methoxy-(polyethyleneglycol)-2000] (DSPE-mPEG2000) (Mean Molecular Weight (MMW)=2,805 g/mol) was purchased from Avanti Polar Lipids (Alabaster, USA). 1,10-dioctadecyl-3,3,30,30-tetramethylindocarbocyanine perchlorate (DiI) was obtained from Introgen (Cergy, Pontoise, France).

Animals

All *in vivo* experiments were carried out on 10–11-week-old Syngeneic Fischer F344 female rats (Charles River Laboratories France, L'Arbresle, France), weighing 160–180 g. Animal care was carried out in strict accordance with French Ministry of Agriculture regulations.

Preparation of LNCs

LNCs were prepared following a phase-inversion process as previously described (6). Briefly, Solutol® HS15 (17% w/w), Lipoid® (1.5% w/w), Labrafac® (20% w/w), NaCl (1.75% w/w) and water (59.75% w/w) were mixed and heated under magnetic stirring up to 85°C. Three cycles of progressive heating and cooling between 60 and 85°C were then carried out to form a microemulsion in the phase-inversion zone (PIZ). A sudden dilution with 28.5% v/v of cold water was then added to the mixture at 70–75°C in order to break the microemulsion system obtained in the PIZ, leading to the formation of stable nanocapsules (20). Images of LNC by using cryo-TEM (transmission electron microscopy) as well as atomic force microscopy were already realized and shown in (21–25). Slow magnetic stirring was then applied to the suspension for 5 min. Final LNC suspensions were filtrated through a Minisart 0.2 µm filter (Sartorius).

To load the anticancer agent into the oily core of LNCs, FcdiOH was first dissolved in Labrafac® under ultrasound at 4% w/w, and the resulting lipophilic phase was then mixed with other components as described above. This procedure provided FcdiOH-LNCs at a high drug concentration of 6.5 mg/g (2% w/w dry weight).

Fluorescent LNCs were obtained by labelling LNCs with DiI fluorochrome (emission wavelength = 549 nm; excitation wavelength = 565 nm). The preparation was performed as previously described (26). Briefly, DiI was dissolved in acetone at 3 mg/mL, and the resulting solution was incorporated in the Labrafac® at 1:30 (v/v). Acetone solvent was then evaporated before mixing with other components.

Post-insertion of DSPE-mPEG2000 onto LNC Surfaces

DSPE-mPEG2000 was incorporated onto the surface of LNCs at the concentration of 10 mM by the post-insertion technique as previously described (13). Briefly, DSPE-mPEG2000 was first dispersed in the water (1/10 total final volume) at 60°C for 15 min. to form micelles. Preformed LNC suspension and DSPE-mPEG2000 micelles were thereafter co-incubated for 2 h at 60°C. The mixture was vortexed every 15 min. and finally quenched in an ice bath for 1 min.

Characterisation of LNCs

The LNCs were diluted 1:100 (v/v) in deionised water, and the measurements of particle size and zeta potential were performed at 25°C. The LNCs were analysed in triplicate for their mean particle diameter, polydispersity index (PdI) and zeta potential using a Malvern Zetasizer® (Nano Serie DTS 1060, Malvern Instruments S.A., Worcestershire, UK).

In Vitro Complement Activation

The complement consumption was evaluated in normal human serum (NHS) (provided by the *Etablissement Français du Sang*, CHU, Angers, France) by measuring the residual haemolytic capacity of the serum complement after contact with the different particles. The technique, according to the procedure described elsewhere (27), consisted in dosing the amount of serum able to haemolyse 50% of a fixed number of sensitised sheep erythrocytes with rabbit anti-sheep erythrocyte antibodies (CH50 unit). Complement activation was presented as a function of the nanoparticle surface area which was calculated as previously described (28). All experiments were performed in triplicate, and a student *t*-test of non-matched samples was used to test the statistical significance of the results.

In Vitro Macrophage Uptake

THP-1 cells (human monocyte/macrophage cell line obtained by ATCC, Manassas, VA, USA) were grown in suspension in a humidified atmosphere containing 5% CO_2 at 37°C in ATCC recommended medium. Cells were cultured in the medium containing 1:1 solution of ATCC recommended medium and 200 nM Phorbol 12-myristate 13-acetate (PMA, Sigma, Saint-Quentin Fallavier, France) for 48 h to allow adherence and differentiation (29). The medium was then aspired, and the cells were subsequently incubated in a new medium for an additional 24 h prior to uptake studies. The cells were harvested and counted using Trypan blue exclusion assay with a haemacytometer. Cells (0.6×10^6/mL) were then placed on a 24-well plate for 24 h.

Dil-labelled LNC suspensions were diluted in MiliQ water to obtain a concentration of particles in suspension of 16.5 mg/g. The culture medium was totally removed 24 h after culture, and the cells were incubated at 4 or 37°C for 1.5 h with 50 µL of previously prepared LNC suspensions and 500 µL medium. The cells were then washed twice with DPBS to remove attached nanoparticles, followed by trypsinisation. After centrifugation, they were re-suspended in a 0.4% (w/v) Trypan Blue solution in DPBS to quench the extracellular fluorescence, thus enabling the determination of the fraction that was actually internalised. The treated samples were subsequently washed twice and analysed by flow cytometry in at least triplicate experiments. For quantification analysis, untreated cells were considered as having 100% fluorescence intensity.

In Vivo Pharmacokinetic Study

Four hundred microlitres of DiI-LNCs or DSPE-mPEG2000-DiI-LNCs were administered into healthy Fischer female rats through the tail vein. Blood samples were collected by cardiac puncture at designated time intervals in a venous blood collection tube (Vacutainer, SST II Advance, 5 mL, Becton Dickinson France SAS, Le Pont-De-Claix, France). One hundred and fifty microlitres of sample plasma harvested after centrifugation at 2,000 g for 10 min. were then put in a black 96-well plate (Greiner Bio-one, Frickenhausen, Germany). DiI fluorescence was measured at emission wavelength of 544 nm with an excitation wavelength of 590 nm by a Fluoroscan (Ascent FL, Thermo Fisher Scientific, Cergy-Pontoise, France). Plasma residual fluorescence was measured from the supernatant of centrifuged blood taken from three rats receiving 400 µL of a physiological saline solution. Fluorescence was expressed in fluorescence units (FU) and was calculated as $FU_{sample}-FU_{residue}$. One hundred percent of fluorescence was considered as the value counted at 1 min post-injection.

Pharmacokinetic data were analysed by non-compartmental calculation from the percentage of the injected dose *versus* time profiles by Kinetica 4.1.1 software (Thermo Fisher Scientific, Villebon-sur-Yvette, France). The half-life was calculated as follows: $T_{1/2}=Log(2)/Lz$, where Lz was determined from linear regression using defined intervals. The trapezoidal rule (linear rule) was applied to calculate the area under the curve (AUC) without extrapolation. The AUC was calculated from the mean % FU values observed from the time of LNC administration plus 24 h.

In Vivo Antitumour Efficacy Study

Tumour Cell Line

Rat 9L gliosarcoma cells were obtained from the European Collection of Cell Culture (Salisbury, UK, N°94110705). The cells were cultured at 37°C/5% CO_2 in Dulbecco modified eagle medium (DMEM) with glucose and L-glutamine (BioWhittaker, Verviers, Belgium) containing 10% foetal calf serum (FCS) (BioWhittaker) and 1% antibiotic and antimycotic solution (Sigma, Saint-Quentin Fallavier, France). On the day of implantation, cells were trypsinised and resuspended into minimal essential medium (EMEM), without FCS or antibiotics, to the final desired concentration.

Subcutaneous Gliosarcoma Model and Therapy Schedule

Animals were manipulated under isoflurane/oxygen anaesthesia. After shaving and disinfection, rats were subcutaneously implanted with 1.5×10^6 9L cells on the right flank. Tumour growth was tracked by regularly measuring the length and width of tumours with a calliper. The tumour volume (V) was estimated by the mathematical ellipsoid formula: $V = (\pi/6) \times (\text{width})^2 \times (\text{length})$.

When tumours reached a calculated average volume of approximately 76 mm^3, the rats were randomised into four groups to ensure that the initial tumour volumes on the day of treatment were not significantly different among groups. Animals were treated (Day 0) by a single intravenous injection of different treatments (400 µL) via the lateral tail vein as follows: physiological saline solution (0.9% NaCl), blank LNCs, FcdiOH-LNCs (2.4 mg/rat) and DSPE-mPEG2000-FcdiOH-LNCs (2.4 mg/rat).

Tumour size was measured twice weekly after the intravenous administration of treatments. At Day 25, rats were sacrificed in a CO_2 chamber, and the tumours were then isolated and weighed. The statistically significant difference in tumour volume and mass among groups was analysed using the two-tail student *t*-test and was considered as significant with $P<0.05$.

Intracranial Gliosarcoma Model and Therapy Schedule

The animals were anaesthetised by an intraperitoneal injection of a mixture of 1:1 solution of ketamine (100 mg/kg body weight) (Clorketam®, Vétoquinol, Lure, France) and xylazine (20 mg/kg body weight) (Rompun®, Bayer, Puteaux, France). Ten microlitres of 10^3 9L cell suspension were stereotaxically implanted for 5 min. into the rat striata using a 10 µL syringe (Hamilton® glass syringe 700 series RN) with a 32G needle (Hamilton®) (8). The cannula coordinates were 1 mm posterior from the bregma, 3 mm lateral from the sagittal suture and 5 mm below the dura (with the incisor bar set at 0 mm).

Six days after 9L cell implantation, the rats were anaesthetised by isoflurane/oxygen inhalation. Rats were randomly treated by intravenous injection (400 µL) with blank LNCs, FcdiOH-LNCs (2.4 mg/rat), or DSPE-mPEG2000-FcdiOH-LNCs (2.4 mg/rat). The untreated control group did not receive any treatment injection.

The experimental rats were weighed every 6 days. To determine rat survival time, criteria for euthanasia were applied. The animals were sacrificed in a CO_2 chamber when they lost 20% of body weight and/or presented seizure, hunched posture and haemorrhaging around the eyes, mouth and nose. The death was recorded as if it occurred on the day following sacrifice and represented the survival time on the Kaplan-Meier curves of gliosarcoma-bearing rats. The statistical significance was estimated from the log-rank test (Mantel-Cox Test) by using StatView software version 5.0 (SAS institute Inc.). Tests were considered as significant with $P<0.05$. The different treatment groups were compared in terms of median and mean survival time in days after 9L cell implantation. The percentage of

increase in survival time (% IST) was determined relative to the median and mean survival times of untreated controls as presented in the following equation:

$$\%\mathrm{IST} = [\mathrm{Median_T}(\mathrm{Mean_T}) - \mathrm{Median_C}(\mathrm{Mean_C})]/\mathrm{Median_C}(\mathrm{Mean_C})$$

where $\mathrm{Median_T}/\mathrm{Mean_T}$ was the median/mean of survival time of the treated group and $\mathrm{Median_C}/\mathrm{Mean_C}$ was the median/mean of survival time of the control group.

RESULTS

Preparation and Characterisation of LNCs

The physicochemical properties of the different kinds of LNCs are presented in Table I. The size distribution of LNCs was unimodal (PdI <0.1). Blank LNCs presented a median diameter of 47.92 ± 0.93 nm with a slightly negative zeta potential (−6.51 ± 1.01 mV). The loading with FcdiOH as well as the DiI fluorochrome into the oily core of LNCs did not affect the particle size or the zeta potential. Because of lipophilicity of FcdiOH (water solubility inferior to 0.001 μg/ml), the filtration through a hydrophilic Minisart 0.2 μm filter after preparation allowed some unincorporated products to be eliminated. The drug loading and encapsulation efficiency, determined by spectrophotometry at 450 nm after dissolving LNCs in a mixture of 22/67/11 (v/v/v) acetone/THF/water solution (as previously described (4)), were calculated by comparing the non-filtrated and filtrated parts of formulation. FcdiOH was well encapsulated in the LNCs at an interesting drug-loading capacity (6.44 ± 0.08 mg/g−2% w/w) with high encapsulation efficiency (98.46 ± 1.25%).

The coating of DSPE-mPEG2000 slightly increased mean particle size but notably decreased the zeta potential (−24.37 to −22.5 mV) owing to the formation of dipoles between PEG molecules and water, as previously described (30).

Complement Consumption and Macrophage Uptake

The *in vitro* complement activation was evaluated by the CH50 method, which measures the activation of the entire complement system (9). This method consists of determining the residual haemolytic capacity of a fixed amount of normal human serum towards 50% of antibody-sensitized sheep erythrocytes in the presence of an increasing nanoparticle surface area. As presented in Fig. 1, all types of tested LNCs, including blank LNCs, FcdiOH-LNCs, DSPE-mPEG2000-FcdiOH-LNCs, demonstrated weak complement activation. Indeed, at a total surface area of approximately 1,000 cm^2, the CH50 unit, consumption remained under 20% for all the LNCs. This value was very low when compared to the positive control, polymethyl methacrylate (PMMA) nanoparticles, which were considered as strong complement activators with a maximal CH50 unit consumption of 100% at a surface of around 300–400 cm^2 (13,27). Among the three types of tested LNCs, the slight increase of the CH50 value of DSPE-mPEG2000-FcdiOH-LNCs could be attributed to their increase in mean particle size, as already observed (21,27).

In order to evaluate the *in vitro* uptake of nanoparticles by macrophages, LNCs were labelled with DiI, and the quantitative uptake was analysed by flow cytometry. The fluorescent intensity of the macrophage population was counted after incubation of the different kinds of LNCs with THP-1 human macrophage cells at 4°C or 37°C. The macrophage uptake of particles at 37°C represents the total uptake, including the adsorption of nanoparticles onto the cell surface and the active cellular endocytosis. At 4°C, endocytosis is inhibited, and only the binding process takes place. By scaling the residual fluorescent intensity of untreated cells to 100%, an increase of about 10–22% in fluorescent intensity was observed for all tested LNCs Fig. 2. Moreover, the macrophage uptake of LNCs by THP-1 cells at 37°C was not significantly different from this at 4°C. This suggested a low *in vitro* phagocytosis of tested LNCs by macrophage cells.

Pharmacokinetics of LNCs in Rats

As shown in the results of physicochemical characteristics, the loading with either FcdiOH or DiI did not alter the mean size or the zeta potential of LNCs owing to their good encapsulation in the oily core of LNCs. Therefore, in this study, DiI fluorochrome was used for tracking conventional

Table I Physicochemical Characteristics of LNC Suspensions

	Mean particle size (nm)	Poly-dispersity PdI	Zeta potential (mV)
Blank LNCs	47.92 ± 0.93	0.038 ± 0.007	−6.51 ± 1.01
FcdiOH-LNCs	46.07 ± 0.11	0.038 ± 0.011	−6.96 ± 0.67
DSPE-PEG-FcdiOH-LNCs	52.85 ± 2.82	0.075 ± 0.008	−24.37 ± 1.93
DiI-LNCs	49.36 ± 0.36	0.045 ± 0.007	−5.68 ± 0.54
DSPE-PEG-DiI-LNCs	54.01 ± 1.18	0.050 ± 0.010	−22.50 ± 1.04

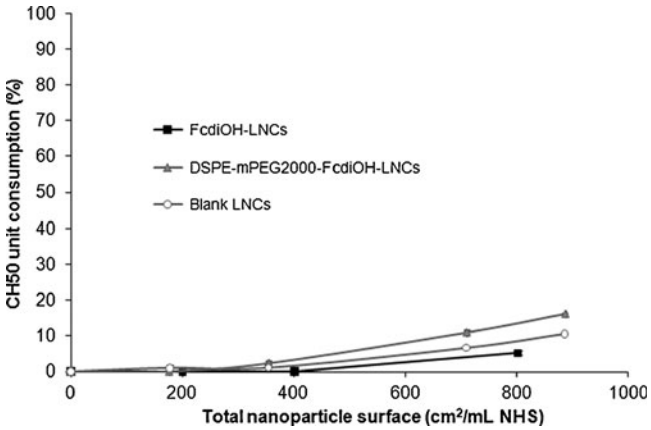

Fig. 1 Complement activation of blank LNCs, FcdiOH-LNCs and DSPE-mPEG2000-FcdiOH-LNCs expressed by % consumption of CH50 unit at 37°C in function of the nanoparticle surface area. Results are represented as mean ± SEM.

LNCs (DiI-LNCs) and PEGylated LNCs (DSPE-mPEG2000-DiI-LNCs), which were considered as being representative of FcdiOH-LNCs and DSPE-mPEG2000-FcdiOH-LNCs.

DiI-LNCs and DSPE-mPEG2000-DiI-LNCs were intravenously administered into healthy Fisher rats. The blood of the rats was collected at 1, 5, 15, 30 min. and 1, 3, 5, 24 h following the injection. Figure 3 illustrates the plasma pharmacokinetic profile of DSPE-mPEG2000-DiI-LNCs compared to DiI-LNCs. A rapid decrease of the injected dose of DiI-LNCs was observed during the first hour post-injection, and only a small fluorescent fraction (<30%) was detected in the plasma at 30 min. after intravenous injection. On the contrary, DSPE-mPEG2000-DiI-LNCs remained at a high level (about 65%) at 3 h post-injection. Finally, approximately 10% of the injected dose of both DiI-LNCs and DSPE-mPEG2000-DiI-LNCs remained in the blood 24 h post-injection.

By calculating with Kinetica 4.1.1 software, the estimated half-life value for DiI-LNCs was 1.95 h with AUC_{last} of 381 (% injected dose/h). DSPE-mPEG2000-DiI-LNCs exhibited a prolonged half-life of 7.82 h with an extended AUC of 623 (% injected dose/h). This result is in accordance with a previous study showing the extended half-life of DSPE-mPEG2000-DNA-LNCs compared to DNA-LNCs (13). The short length of PEG chains can explain the rapid elimination of conventional LNCs from blood circulation, despite their weak complement activation and low macrophage uptake in *in vitro* studies (11,27).

Anti-tumour Activity of FcdiOH-LNCs on a Subcutaneous Gliosarcoma Model

This study aimed at investigating whether DSPE-mPEG2000-FcdiOH-LNCs could accumulate in a subcutaneous, 9L gliosarcoma tumour after an intravenous injection in rats and then exhibit the intrinsic anti-tumour activity of FcdiOH in comparison with conventional FcdiOH-LNCs.

The results presented in Fig. 4 show tumour growth during experiment (a) and the mass of isolated tumours (b) after sacrificing the rats in a CO_2 chamber at the end of the study (Day 25). Subcutaneous tumours in control groups receiving either 0.9% NaCl or blank LNCs grew very quickly and reached a volume of about 3,000 mm^3 and 2,300 mm^3, respectively. These corresponded to a tumour mass of 2,400 and 1,850 mg, respectively at Day 25. The intravenous administration with FcdiOH-LNCs significantly lowered tumour volumes as well as the tumour mass when compared to the saline control group ($P<0.05$). However, tumour volumes always increased during the experiments. In fact, by Day 25, tumour volume reached about 1,300 mm^3 with a mass of 1,050 mg. On the contrary, DSPE-mPEG2000-FcdiOH-LNC treatment strongly inhibited tumour growth. This treatment did not only reduce the tumour volume but also nearly eradicated the tumour, with a dramatically decreased tumour mass down to 33 mg by the end of the study.

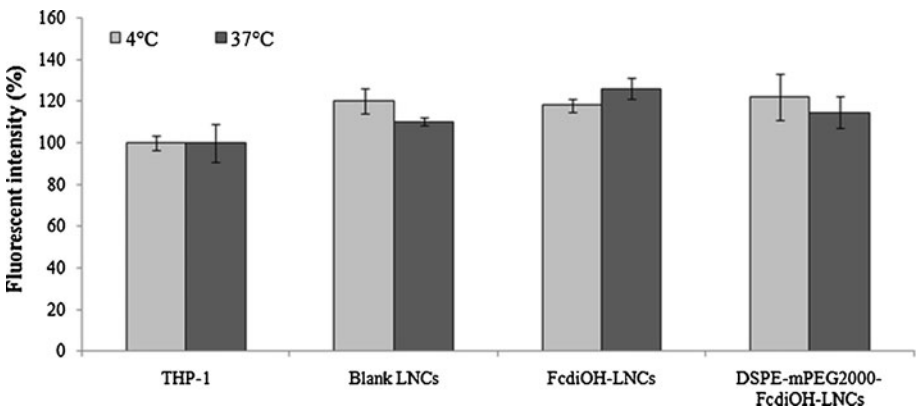

Fig. 2 Quantification of *in vitro* macrophage uptake of blank LNCs, FcdiOH-LNCs and DSPE-mPEG2000-FcdiOH-LNCs. Fluorescent intensity was measured 24 h after incubation of DiI-labelled LNCs with THP-1 cells. The residual fluorescence of THP-1 cells alone was scaled to 100%. Results are represented as mean ± SEM.

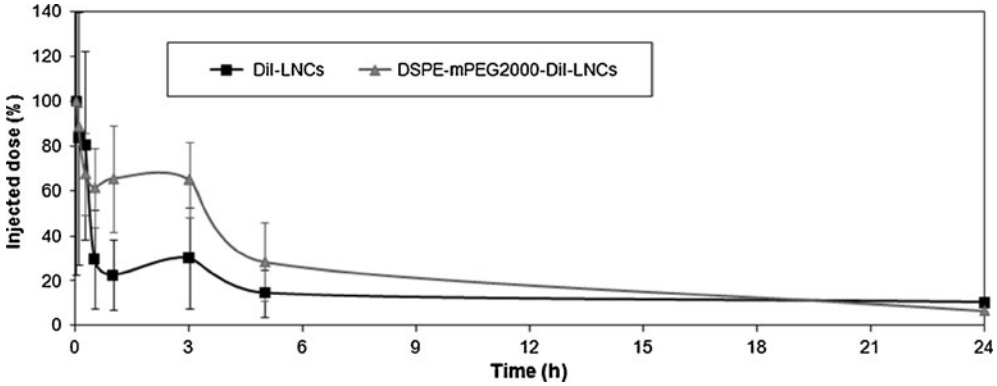

Fig. 3 Plasma concentration-time profile of conventional DiI-LNCs and DSPE-mPEG2000-DiI-LNCs during 24 h after a single intravenous injection into healthy female Fisher rats. Data are represented as mean ± SEM.

Anti-tumour Activity of FcdiOH-LNCs on an Intracranial Gliosarcoma Model

The promising results of anti-tumour activity of FcdiOH-LNCs and DSPE-mPEG2000-FcdiOH-LNCs led to the investigation of their efficacy in an orthotopic 9L gliosarcoma model. In this study, tumour cells were implanted into the striata of experimental rats. On Day 6 after cell implantation, they were treated with FcdiOH-LNCs and DSPE-mPEG2000-FcdiOH-LNCs at the same dose used in the subcutaneous 9L gliosarcoma model (400 µL, 2.4 mg/rat).

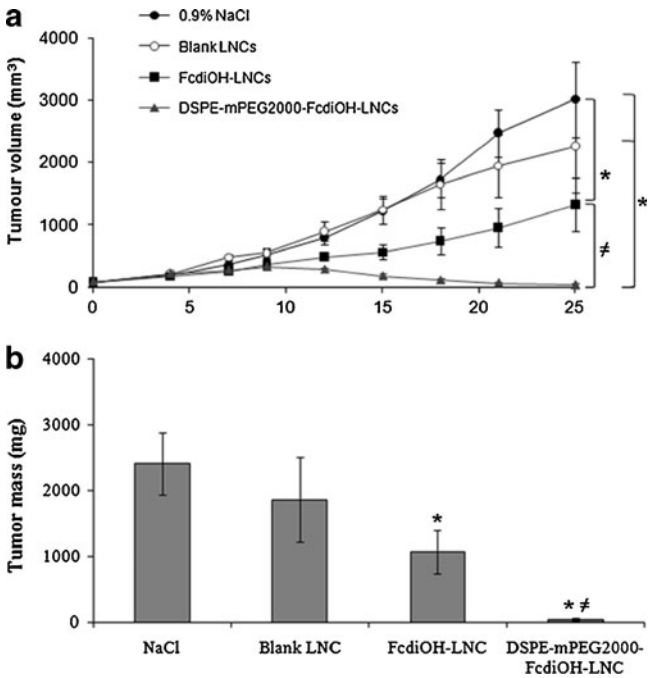

Fig. 4 Anti-tumoural effect of FcdiOH-LNCs and DSPE-mPEG2000-FcdiOH-LNCs after a single intravenous treatment as compared to control groups receiving 0.9% NaCl solution or blank LNCs in a subcutaneous 9L gliosarcoma rat model. Treatment was initiated when the tumour volume reached about 76 mm^3 (Day 0). Tumour growth (**a**) was estimated by measuring twice weekly the length and width of tumours with callipers; at Day 25 post-treatment, the tumours were isolated after sacrificing the rats to determine tumour mass (**b**). Data are represented as mean ± SEM. * and ≠ express significant differences ($P < 0.05$) as compared to control groups and FcdiOH-LNC treatment, respectively.

The survival data of the experimental rats are summarised in Table II, and Kaplan–Meier survival plots are shown in Fig. 5. Intracranial 9L gliosarcoma-bearing rats exhibited normal behaviour and no signs of illness for at least 2 weeks after implantation. Indeed, the recorded weights demonstrated their continuous weight gain (data not shown). From Day 18, the general condition of untreated rats worsened rapidly, with poor grooming, decrease in activity and reflexes, and rapid weight loss. Untreated control rats were sacrificed from Day 22 to Day 26 after cell implantation. The median and mean survival time for the untreated control group was 25 and 25.13 ± 1.25 days, respectively. The administration of drug-free LNCs (blank LNCs) resulted in a median survival time of 26.5 days, and there was no significant difference when compared to the untreated control group. By slightly increasing the median survival time to 27.5 days, treatment with FcdiOH-LNCs was statistically different from the untreated control group. Finally, DSPE-mPEG2000-FcdiOH-LNCs improved the survival time of treated rats with median and mean survival times of 28 days and 28.75 ± 1.58 days, respectively, leading to a significant difference with the untreated control group as well as the blank LNC-treated group, but not with the FcdiOH-LNC-treated group ($P = 0.1914$).

DISCUSSION

Over the past few decades, long-circulating nanoparticles, so-called "stealth" nanoparticles, have been attracting increasing interest as a new platform for targeting drug delivery, especially in chemotherapy. In particular, the modification of nanoparticle surfaces by attaching PEG moieties has illustrated an increased circulation time after intravenous injection in a great number of examples (10,31–33). This allows the passive targeting for drug delivery to the tumour tissues by the EPR effect. Taking the advantages of PEGylated nanoparticles into account, in

Table II Survival Time of Intracranial, 9L Gliosarcoma-Bearing Rats that Received an Intravenous Injection of Blank LNCs, FcdiOH-LNCs or DSPE-mPEG2000-FcdiOH-LNCs at Day 6 After Cell Implantation, Compared to an Untreated Control Group

Treatment	*n*	Survival time (days)			% IST		*P* value *vs* untreated control
		Range	Median	Mean ± SD	Median	Mean	
DSPE-mPEG2000-FcdiOH-LNCs (2.4 mg/rat)	8	27–32	28	28.75 ± 1.58	12	14.41	0.0001
FcdiOH-LNCs (2.4 mg/rat)	8	26–30	27.5	27.75 ± 1.28	10	10.43	0.0011
Blank LNCs	4	24–27	26.5	26.00 ± 1.41	6	3.46	0.2157
Untreated control	8	23–27	25	25.13 ± 1.25	–	–	–

% *IST* percentage of increase in survival time relative to that of the untreated control

this study, long chains of PEG linked to the lipid anchor of distearoylphosphatidyl-ethanolamine (DSPE-mPEG2000) were incorporated on the surface of LNCs that were conventionally coated with PEG 660.

In vitro complement activation and macrophage uptake provided predictive indicators for *in vivo* long-circulating behaviour, according to the stealth properties of the nanoparticles (34). Results from *in vitro* studies showed that all kinds of tested LNCs, including blank LNCs, FcdiOH-LNCs and DSPE-mPEG2000-FcdiOH-LNCs, presented a weak complement activation as well as a low uptake by THP-1 macrophage cells. However, when intravenously administrated into healthy rats, conventional LNCs and pegylated LNCs exhibited different pharmacokinetic profiles. DSPE-mPEG2000-DiI-LNCs presented a 4-fold longer half-life and 1.65-fold larger AUC than DiI-LNCs, thus providing more chances to reach tumour tissues through the EPR effect thanks to their long-circulating property.

Therefore, the potential of nanocarriers for the delivery of FcdiOH to the tumour was tested on an ectopic gliosarcoma rat model, *i.e.* subcutaneously implanted 9L tumours after a single intravenous injection. The administration of FcdiOH-LNCs significantly reduced the tumour volume as well as tumour mass, showing the *in vivo* effectiveness of FcdiOH in inhibiting tumour growth. The profile of tumour volume *versus* time was comparable to that of the intratumoural injection of FcdiOH-LNCs on the same ectopic gliosarcoma model in rats as previously observed (4). The remarkable thing in the present work is that DSPE-mPEG2000-FcdiOH-LNC treatment nearly eradicated subcutaneous tumours following a single intravenous injection. This indicates, on one hand, the accumulation of DSPE-mPEG2000-FcdiOH-LNCs in the tumour tissues, as already witnessed (13), showing the potential of such targeting nanocarriers for drug delivery to tumour tissues. On the other hand, the *in vivo* intrinsic antitumoural activity of FcdiOH was strengthened. Indeed, a large number of studies in the literature have usually dealt with repeated injections (35–38) or a combination treatment (39) to achieve significant efficacy.

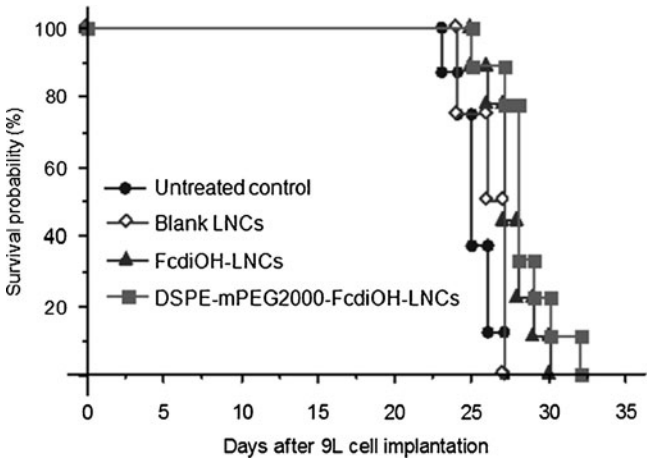

Fig. 5 Kaplan-Meier curves plot the survival times of experimental rats with an intracranial 9L gliosarcoma model. 9L gliosarcoma cells were implanted intracranially into the striata of rats, followed by the intravenous administration of blank LNCs, FcdiOH-LNCs or DSPE-mPEG2000-FcdiOH-LNCs at Day 6 after cell implantation. Untreated control rats did not received any treatment.

In our more clinically relevant orthotopic gliosarcoma model, the single intravenous administration of FcdiOH-LNCs and DSPE-mPEG2000-FcdiOH-LNCs statistically improved the survival of treated rats, despite their moderate increase in median survival time as compared to the untreated control (8–12%). The increased survival time of animals is comparable with that of the local treatment by CED with FcdiOH-LNCs (median survival time of 27 days) as previously reported (8). This suggests that the systemic treatment of coated LNCs is as efficient as local treatment by CED.

Owing to the presence of the blood-brain barrier, drug delivery to the brain by systemic routes remains challenging to achieve an effective response to treatment (40,41). In the case of brain tumours, the blood-brain barrier or blood-tumour barrier become abnormal because of defects in inter-endothelial tight junctions that correlate with increasing malignancy in human gliomas (14). Therefore, the systemic delivery of drugs for treatment of brain cancer may become possible. Brigger *et al.* have shown that

following an intravenous injection of PEG-PHDCA NPs or PHDCA NPs into intracranial 9L gliosarcoma-bearing rats, both types of nanospheres were preferentially accumulated in the 9L gliosarcoma of rats, whereas no accumulation of these nanospheres was observed for healthy animals at the intracranial injection site of NaCl (18). However, only a small fraction of the injected dose was detected in the tumour tissues 8 h post-injection (maximal concentration about 0.22% per gram of tumour tissues). Therefore, although the blood-brain barrier was disrupted by the presence of a brain tumour, the efficacy of drugs administered by a systemic injection was still limited by poor penetration through this barrier (42). Moreover, many solid tumours exhibit a high interstitial fluid pressure, which represents also a barrier to transcapillary transport and inhibits the homogeneity of therapeutic agent distribution in tumour tissues (43).

In addition, due to organ-specific upregulation of angiogenic factors, predominantly vascular endothelial growth factor (VEGF) that promotes the formation of new blood vessels to support tumour growth (44,45), biological differences make the orthotopic brain tumour more difficult to treat. Therefore, promising results from a single intravenous injection in the present study demonstrate the potential of the tested anticancer agent as well as the drug nanocarriers for the treatment of brain cancer. Moreover, as previously reported, the anti-tumoural effect of FcdiOH on the orthotopic 9L gliosarcoma model was observed in a dose-dependent manner (8). As a consequence, a repeated-injection regimen should be planned to increase the injected drug dose and, consequently, further enhance antitumour efficacy.

CONCLUSION

Results from the present study show that coating DSPE-mPEG2000 to the surface of LNCs confers long-circulating properties leading to the passive accumulation of PEGylated LNCs at the tumour site. Together with the antitumour activity of FcdiOH, this allows an improvement of the beneficial effect of this drug and opens a new prospect for the application of these bio-organometallic drugs in cancer chemotherapy via systemic administration.

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