Dose effect activity of ferrocifen-loaded lipid nanocapsules on a 9L-glioma model

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Abstract

Ferrociphenol (Fc-diOH) is a new molecule belonging to the fast-growing family of organometallic anti-cancer drugs. In a previous study, we showed promising *in vivo* results obtained after the intratumoural subcutaneous administration of the new drug-carrier system Fc-diOH-LNCs on a 9L-glioma model. To further increase the dose of this lipophilic entity, we have created a series of prodrugs of Fc-diOH. The phenol groups were protected by either an acetyl (Fc-diAc) or by the long fatty-acid chain of a palmitate (Fc-diPal). LNCs loaded with Fc-diOH prodrugs have to be activated *in situ* by enzymatic hydrolysis. We show here that the protection of diphenol groups with palmitoyl results in the loss of Fc-diOH *in vitro* activity, probably due to a lack of *in situ* hydrolysis. On the contrary, protection with an acetate group does not affect the strong, *in vitro*, antiproliferative effect of ferrocifen-loaded-LNCs neither the reduction of tumour volume observed on an ectopic model, confirming that acetate is easily cleaved by cell hydrolases. Moreover, the cytostatic activity of Fc-diOH-LNCs is confirmed on an orthotopic glioma model since the difference in survival time between the infusion of 0.36 mg/rat Fc-diOH-LNCs and blank LNCs is statistically significant. By using LNCs or Labrafac[®] to carry the drug, a dose-effect ranging from 0.005 to 2.5 mg of Fc-diOH per animal can be evidenced.

1. Introduction

Bioorganometallic chemistry is a growing, multidisciplinary field which encompasses the synthesis and study of organometallic complexes of biological importance (Jaouen, 2006). One of the most promising applications of bioorganometallic chemistry is in the area of drug development. Recently, it was discovered that grafting a ferrocenyl unit onto a tamoxifen skeleton allowed the preparation of a new and promising class of ferrocifen-type, anticancer drug candidates (Top et al., 2003, Hillard et al., 2006a). These new molecules, called ‘ferrocifens’, by analogy to tamoxifen, have the advantage of exhibiting dual functionality: not only do they possess endocrine-modulating properties, but also cytotoxic activity (Vessieres et al., 2005). Unfortunately, these molecules suffer from poor bioavailability because they contain highly hydrophobic phenol groups. This may limit both their potential for future clinical application and also the study of their activity under conditions that are similar to the cellular medium. In this context, we have previously shown that it is possible to formulate these molecules in lipid nanocapsules (LNCs) according to an organic, solvent-free process recently developed in our laboratory (Heurtault et al., 2002). LNCs loaded with the diphenol compound Fc-diOH can be obtained with a high drug-loading capacity. It was also found that cytostatic activity was conserved after their encapsulation in LNCs on 9L-glioma cells with an IC_{50} value of 0.6 μ M while Fc-diOH-LNCs were harmless on healthy brain cells up to a concentration range of 10 μ M (Allard et al., 2008a). Promising *in vivo* results were also obtained after the intratumoural administration of this new drug-carrier in a subcutaneously injected 9L model, as it dramatically reduced the tumour mass and glioma volume (Allard et al., 2008b).

In this study, the objective was to test these molecules in an orthotopic model on rats. For that purpose, LNC formulations loaded with ‘ferrocifen’ molecules had to be sufficiently concentrated to exert their antitumoural effect. Indeed, the volume infused by brain administration is limited because of a problem of clinical tolerance linked to intracranial pressure (Allard et al., 2009).

We thought that an increase of the lipophilicity of Fc-diOH obtained by protection with long or short alkyl chains could enhance the quantity of drug encapsulated in the oily core of nanocapsules, thus increasing the quantity of drug available for brain administration. To

further evaluate this hypothesis, we created two Fc-diOH prodrugs carrying acetyl (Fc-diAc) or palmitoyl (Fc-diPal) chains as protecting groups (Fig. 1). It has been hypothesised that the Fc-diOH mechanism of action could be linked to the oxidation of phenol groups leading to the formation of quinone methide, which is known to react with nucleophiles such as glutathione, thus eventually leading to cell death (Hillard et al., 2006b). This transformation can only occur with a particular structural motif, where the ferrocenyl group is located on carbon 2 of the but-1-ene group, the phenol group resides on carbon 1, and a conjugated π -system exists between the ferrocenyl and phenol groups (Hillard et al., 2007). It seems clear that the initiation of Fc-diAc and Fc-diPal will require *in situ* enzymatic hydrolysis. A previous study performed with Fc-diAc on hormone-independent breast cancer cells (MDA-MB-231) showed that its activity was comparable to that of Fc-diOH, suggesting that hydrolases available in the living cells transformed the diphenol ester function *in situ* (Heilmann et al., 2008). However, it should be kept in mind that the brain is not a suitable environment to prime the oxidative process responsible for Fc-diOH cytotoxicity. Indeed brain, spinal cord and adrenal glands have the highest ascorbate concentrations of all body tissues, as well as the greatest retention capacities, which means that the brain environment is a very reducing medium (Rice, 2000). In the present study, we first evaluated the possibility to encapsulate the molecules in LNCs by dissolving the prodrugs in the triglyceride core of the capsules, then, the antiproliferative action of the prodrugs was compared to that of Fc-diOH on 9L-glioma cells. Molecules selected from their *in vitro* cytotoxicities were then tested for *in vivo* experiments on a 9L ectopic tumour model on Fischer rats. Finally, the dose effect was investigated on a 9L brain tumour model by administrating ‘ferrocifen’ molecules by stereotaxy or by convection-enhanced delivery.

Fig. 1. Chemical formulae of ferrocifen molecules; 2-ferrocenyl-1,1-bis(4-hydroxyphenyl)-but-1-ene called Fc-diOH (MMr = 424.4 g mol⁻¹) and its prodrugs Fc-diAc (MMr = 508.4 g mol⁻¹) and Fc-diPal (MMr = 901.4 g mol⁻¹).

2. Materials and methods

2.1. Materials

A ferrocenyl diphenol compound (2-ferrocenyl-1,1-bis(4-hydroxyphenyl)-but-1-ene), named Fc-diOH, was prepared by McMurry coupling (Jaouen et al., 2000). Fc-diAc (2-ferrocenyl-1,1-bis(4-acetoxyphenyl)-but-1-ene) was prepared according to a previously described protocol (Heilmann et al., 2008) and Fc-diPal (2-ferrocenyl-1,1-bis(4-palmitoylphenyl)-but-1-ene) was prepared according to the procedure described above. In portions and under stirring, a suspension of 60% sodium hydride in oil (0.3 g, 12.5 mmol) was added to a solution of diphenol Fc-diOH (2.12 g, 5 mmol) in dry THF (30 mL). The stirring was maintained for 10 min and the acyl chloride was slowly added (11 mmol: 3.024 g for palmitoyl chloride). The reaction mixture was stirred for 3 h and then ethanol (5 mL) was added. After 1 h, the mixture was poured into water, CH₂Cl₂ was added, and the mixture was decanted. The aqueous phase was extracted with CH₂Cl₂, the combination of organic layers was dried under magnesium sulphate, concentrated under reduced pressure, and chromatographed with CH₂Cl₂. For Fc-diPal: Yield 91%. Anal. Calc. for C₅₈H₈₄FeO₄: C, 77.30; H, 9.39. Found: C, 77.22; H, 9.41.

For lipid nanocapsule (LNC) preparation, the lipophilic Labrafac[®] CC (caprylic-capric acid triglyceride) was kindly provided by Gattefosse S.A. (Saint-Priest, France). Lipoid[®] S75-3 (soybean lecithin at 69% phosphatidylcholine) and Solutol[®] HS15 (a mixture of free polyethylene glycol 660 and polyethylene glycol 660 hydroxystearate) were a gift from Lipoid GmbH (Ludwigshafen, Germany) and BASF (Ludwigshafen, Germany), respectively. NaCl was obtained from Prolabo (Fontenay-sous Bois, France). Deionised water was obtained from a Milli-Q plus system (Millipore, Paris, France).

2.2. Preparation of ferrocifen-loaded LNCs

Lipid nanocapsules were prepared according to a previously described original process (Heurtault et al., 2002). In order to obtain LNC, 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 to 85 °C. Three cycles of progressive heating and cooling between 85 and 60 °C were then carried out and followed by an irreversible shock induced by dilution with 2 °C deionised water added when the mixture was at 70–75 °C. To formulate

ferrocifen-loaded LNCs, the first step consisted in dissolving Fc-diOH, Fc-diAc and Fc-diPal in the triglyceride phase (Labrafac[®]) of the formulations using ultrasound. The amount of each prodrug encapsulated in LNCs was calculated to be equivalent in Fc-diOH molecules after hydrolysis of the lipophilic diacetic or dipalmitoyl chains. For *in vitro* experiments, the volume of water for LNC dilution was the same (70% v/v) for all formulations and the amount of ferrocifen molecules solubilised in triglycerides (Labrafac[®]) was 1.7, 2.0 and 3.5% w/w for Fc-diOH, Fc-diAc and Fc-diPal respectively. For a drug load of 1 mg/g for Fc-diOH-LNCs, the prodrug loadings were about 1.2 and 2.1 mg/g for Fc-diAc and Fc-diPal respectively. To formulate Fc-diOH-LNCs 6.5 mg/g, two parameters were changed: the amount of Fc-diOH solubilised in triglyceride (4% w/w) and the volume of cold water (28.5% v/v) added for dilution (Allard et al., 2008b).

2.3. Characterisation of the LNC formulations

2.3.1. Particle size and zeta potential

The nanocarriers were analysed for their size and charge distribution using a Malvern Zetasizer[®] Nano Series DTS 1060 (Malvern Instruments S.A., Worcestershire, UK). The nanocarriers were diluted 1:100 (v/v) in deionised water in order to assure a convenient, scattered intensity on the detector.

2.3.2. LNC drug payload and encapsulation efficiency

Because of the orange colour of the ferrocenyl tamoxifen molecules, the payload was determined by spectrophotometry at 450 nm after dissolving LNCs in solvent mixtures as described below. Part of the formulation of each batch was filtrated using a Minisart[®] 0.1 µm filter (Sartorius). Three samples of each batch of ferrocifen-loaded LNCs (filtrated and non-filtrated) were prepared by dissolving 250 mg of LNCs in 2.25 mL of 22/67/11 (v/v/v) acetone/THF/water solution. Quantification was achieved by comparing the absorbency of ferrocenyl derivative samples to a calibration curve made with blank nanocarriers and a ferrocifen ethanol/THF/water solution. The mean drug payloads (mg of drug per gram of LNC dispersion) and encapsulation efficiency (%) were calculated.

2.4. Cell experiments

2.4.1. Cell culture

Rat 9L gliosarcoma cells were obtained from the European Collection of Cell Culture (Salisbury, UK, No. 94110705). The cells were grown at 37 °C/5% CO₂ 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).

2.4.2. Cytotoxicity evaluation of ferrocifen-loaded LNCs

After 96 h, the cell survival percentage was estimated by the MTT survival test. 40 µL of MTT solution at 5 mg/mL in PBS was added to each well, and the plates were incubated at 37 °C for 4 h. The medium was removed and 200 µL of acid-isopropanol 0.06N was added to each well and mixed to completely dissolve the dark blue crystals. The optical density values (OD) were measured at 580 nm for blue intensity and at 750 nm for turbidity using a multiwell-scanning spectrophotometer (Multiskan Ascent, Labsystems SA, Cergy-pontoise, France). The maximal absorbance was determined by incubating cells with free media and was considered as 100% survival (OD_{control}). The cell survival percentage was estimated according to Eq. (1). Each experiment was conducted twice with at least 6 repeated samples.

$$\text{Cell survival}(\%) = \frac{\text{OD}_{580\text{nm}} - \text{OD}_{750\text{nm}}}{\text{OD}_{\text{control } 580\text{nm}} - \text{OD}_{\text{control } 750\text{nm}}} \times 100 \quad (1)$$

2.5. Animal study

2.5.1. Animals and anaesthesia

Syngeneic Fischer F344 female rats weighing 160–175 g were obtained from Charles River Laboratories France (L'Arbresle, France). All experiments were performed on 10 to 11-week old female Fisher rats. The animals were anaesthetised with an isofluorane/oxygen gas mixture for ectopic models (subcutaneous xenograft) and with an intraperitoneal injection of 0.75–1.5 mL/kg of a solution containing 2/3 ketamine (100 mg/mL) (Clorketam[®], Vétquinol, Lure, France) and 1/3 xylazine (20 mg/mL) (Rompun[®], Bayer, Puteaux, France) for orthotopic models. Animal care was carried out in strict accordance to French Ministry of Agriculture regulations.

2.5.2. An ectopic xenograft model

A cultured tumour monolayer was detached with trypsin-ethylene diamine tetraacetic acid, washed twice with EMEM without FCS or antibiotics, counted, and resuspended to the final concentration desired. For tumour growth analysis, animals received subcutaneous injections (s.c) of 1.5×10^6 9L cells into the right thigh. On Day 6 after cell injection, rats implanted with 9L cells were treated by an intratumoural (i.t) single injection (400 μ L) of different treatments. Group 1 was injected with physiological saline solution (control; $n = 7$ animals), group 2 received blank LNCs ($n = 7$ animals), group 3 received Fc-diOH-loaded LNCs 1 mg/g (2.5 mg/kg; $n = 8$ animals) and group 4 was treated with Fc-diAc-loaded LNCs 1.2 mg/g (2.5 mg/kg equivalent Fc-diOH; $n = 8$ animals). The length and width of each tumour were regularly measured using a digital caliper, and tumour volume was estimated with the mathematical ellipsoid formula given in Eq. (2).

$$\text{Volume}(V) = \left(\frac{\pi}{6}\right) \times \text{width}^2(l) \times \text{length}(L) \quad (2)$$

2.5.3. An orthotopic xenograft model

For intracranial implantation, 10 μ L of 10^3 9L cell suspension were injected into the rat striatum at a flow rate of 2 μ L/min using a 10 μ L syringe (Hamilton[®] glass syringe 700 series RN) with a 32G needle (Hamilton[®]). For that purpose, rats were immobilised in a stereotaxic head frame (Lab Standard Stereotaxic; Stoelting, Chicago, IL). A sagittal incision was made through the skin and a burr hole was drilled into the skull with a twist drill. 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). The needle was left in place for 5 additional minutes to avoid expulsion of the suspension from the brain during removal of the syringe, which was withdrawn very slowly (0.5 mm/min).

On Day 6, animals were treated by simple stereotaxy (infusion volume = 10 μ L) or by convection-enhanced delivery (CED) (infusion volume = 60 μ L) (Allard et al., 2008a). Infusions were performed with LNC suspensions or Labrafac[®] solutions at the coordinates of the tumour cells at the depth of 5 mm from the brain surface using a 10 μ L Hamilton[®] syringe with a 32G needle. The groups were as follows: (A) stereotaxy of Fc-diOH-LNCs 1 mg/g (0.005 mg/rat; $n = 7$); (B) CED of Fc-diOH-LNCs 6.5 mg/g (0.36 mg/rat; $n = 8$); (C) CED of

Fc-diOH-Labrafac[®] 40 mg/g (2.5 mg/rat; $n = 7$), (D) stereotaxy of blank LNCs ($n = 7$), (E) CED of blank LNCs ($n = 8$), (F) CED of Labrafac[®] ($n = 8$), (G) control group without treatment but with the same anaesthesia ($n = 9$).

2.6. Statistical analysis

Data from *in vitro* experiments are presented as a mean $\pm$ SD and statistical analysis among groups was conducted with the two-tailed Student *t*-test ($p < 0.05$ was considered to be statistically significant). The Kaplan–Meier method was used to plot animal survival. Statistical significance was calculated using the log-rank test (Mantel–Cox Test). StatView software version 5.0 (SAS institute Inc.) was used for that purpose and tests were considered as significant with p values < 0.05 . The different treatment groups were compared in terms of range, median and mean survival time (days), long term survivors (%) and increase in survival time (IST_{median} and IST_{mean} %).

3. Results and discussion

3.1. Preparation and characterisation of ferrocifen-loaded LNCs

By solubilising ferrocifen molecules in triglycerides (Labrafac[®]) and by applying the phase inversion process on all constituents, lipid nanocapsules loaded with ferrocenyl compounds were prepared. With this process, we were able to obtain a scale of particle sizes from 20 to 100 nm, depending on the proportions of the excipients (Heurtault et al., 2003). We chose to work with the proportions of triglycerides, lecithin, salted-water and hydroxy stearate of poly(ethylene glycol) leading to the formulation of 50 nm-sized nanocapsules. Indeed, the size of distribution of various nanoparticulate carriers has already been shown to influence their application. For brain administration, we previously described that the ideal nanocarrier should be less than 100 nm in diameter (Allard et al., 2009) because they have to diffuse in the extracellular brain space (ECS) which has been estimated between 35 and 64 nm in diameter in the normal rat brain (Thorne and Nicholson, 2006). LNCs loaded with Fc-diOH or its prodrugs had sizes from 50.7 to 60.1 nm, depending on the encapsulated molecule (Table 1). A slight increase of size was observed as the molecular weight of the molecule increased. In fact, in order to obtain equivalent quantities of diphenol molecule Fc-diOH, the quantity of Fc-diAc and Fc-diPal was enhanced to obtain drug load equivalents of 1.2 and 2.1 mg/g

respectively instead of 1 mg/g for Fc-diOH. As the quantity of Labrafac[®] remained unchanged throughout all the formulations, and as the average diameter is known to increase slightly with the proportion of the oily core in the capsule, this could explain the difference in size observed. Nevertheless, all batches were monodispersed as the polydispersity index still remained inferior to 0.13.

Table 1. Physicochemical characteristics of blank and ferrocifen-loaded LNCs.

		Mean particle size (nm)	Polydispersity PDI	Zeta potential (mV)	Encapsulation rate
Blank LNCs		50.7 ± 0.2	0.087 ± 0.009	-10.0 ± 4.1	–
Fc-diOH	LNCs	51.3 ± 0.6	0.093 ± 0.006	-11.1 ± 1.6	98.1 ± 1.7
1 mg/g					
Fc-diAc	LNCs	54.2 ± 2.9	0.084 ± 0.020	-14.0 ± 6.3	96.2 ± 2.9
1.2 mg/g					
Fc-diPal	LNCs	60.1 ± 0.3	0.125 ± 0.006	-17.4 ± 6.7	97.9 ± 0.5
2.1 mg/g					

Drug loading for each formulation was calculated as an equivalent in Fc-diOH molecules.

Ferrocifen-loaded LNCs were also characterised in terms of their surface charge. Zeta potential values ranged from -10.0 to -17.4 mV. With a zeta potential value of -11.1 ± 1.6 mV, which was very close to blank ones (-10.0 mV), we could conclude that the diphenol molecule was mainly contained in the oily core of the LNCs. PEG repartition on LNC surface was not perturbed (Vonarbourg et al., 2005) which means that the entrapment of Fc-diOH was efficient. This was confirmed by high values of encapsulation efficiency of above 98%. The encapsulation of Fc-diOH prodrugs slightly affected the external charge of the nanocapsules with values of -14.0 ± 6.3 and -17.4 ± 6.7 mV. This could mean that the PEG repartition on LNC surfaces might change after encapsulation due to the fact that the pegylated surfactant (Solutol[®]) amounts remained constant, whereas the oily core of LNCs increased. Nevertheless, Fc-diOH prodrugs were well-encapsulated in LNCs with encapsulation efficiency of beyond 95% (Table 1). As expected, the prodrugs were all the more soluble in triglycerides as the length of the alkyl chains increased. As with other hydrophobic drugs (Lamprecht and Benoit, 2006, Malzert-Freon et al., 2006), ferrocifen encapsulation in LNCs was an easy and low-energy emulsification method which avoided the use of organic solvents.

3.2. Cell line experiments

Fc-diOH prodrugs were tested *in vitro* on a survival assay on 9L-glioma cell cultures. In a previous study, we showed that the activity of Fc-diOH was totally recovered *in vitro* after encapsulation and that Fc-diOH-loaded LNCs showed interesting cytotoxic effects on 9L-glioma cells with an IC_{50} of 0.6 $\mu\text{mol/L}$ (Allard et al., 2008b). Conversely, at the same concentration range, Fc-diOH-loaded LNCs showed much reduced toxicity on astrocytes which can be considered as normal brain cells (Allard et al., 2008b). The goal of this work was to compare the activity of Fc-diOH-LNCs versus the activity of Fc-diAc-LNCs and Fc-diPal-LNCs which are expected to be activated by *in situ* hydrolysis enzymes. The results of cytotoxicity survival tests showed that the profiles obtained for Fc-diAc-LNCs and Fc-diOH-LNCs were similar (Fig. 2). In fact, cell survival percentages were about 100% for the lowest concentrations tested (0.001–0.01 $\mu\text{mol/L}$) and near to zero for concentrations above 10 $\mu\text{mol/L}$, with an IC_{50} value of about 0.6 $\mu\text{mol/L}$. Moreover, Fc-diOH or Fc-diAc-loaded LNCs demonstrated cytotoxic activity on 9L cells 150-fold more than on blank LNCs. The activity of Fc-diAc was unchanged compared to Fc-diOH drug which means that the hydrolysis of the acetyl chains can take place in *in vitro* conditions. It could possibly suggest that enzymes in the living cells were able to hydrolyse the ester functions of Fc-diAc to generate the dihydroxyl derivative Fc-diOH *in situ*. This result has already been described in other systems such as ester-estrogen cleavage (Barnes et al., 2004) and the activation of fluorescent probes (Bartos, 2006). Moreover, the encapsulation of the drug did not prevent hydrolysis. As the non-encapsulated acetylated prodrug precipitated in culture medium, this argues for the essential role of nanocapsules for the Fc-diAc uptake. For that reason, LNCs are interesting carriers allowing the cell assimilation of lipophilic drugs. On the contrary, a reduced toxic effect was noted for Fc-diPal-LNCs on 9L cells. Indeed, IC_{50} values were about 20 $\mu\text{mol/L}$ for Fc-diPal-LNCs whereas the incubation of blank LNCs at the same excipient concentration gave IC_{50} values of around 60 $\mu\text{mol/L}$. But the non encapsulated Fc-diPal prodrug caused no cytotoxic effects at any of the tested concentrations (data not shown). Actually, the molecule precipitated in the culture medium and, consequently, could not be assimilated by the cells. It means that Fc-diPal-LNCs were internalized by 9L cells. The lack of cytotoxicity observed for Fc-diPal-LNCs could be attributed only to a lack of efficient *in situ* hydrolysis. For this reason, *in vivo* experiments with this molecule were not performed.

Fig. 2. Cell survival test of 9L cells after 96 h of treatment with ferrocifen-loaded LNCs at concentrations from 0.01 to 100 $\mu\text{mol/L}$ equivalent Fc-diOH. Blank LNCs are tested with the same excipient concentrations as for Fc-diOH-loaded LNCs. Blank LNCs, Fc-diOH-LNCs 1 mg/g, Fc-diAc-LNCs 1.2 mg/g and Fc-diPal-LNCs 2.1 mg/g were diluted in culture medium. Data are expressed as the mean of six wells repeated twice $\pm$ SD ($n = 2$ in 6 wells).

3.3. Ectopic model—subcutaneous administration

A 9L, subcutaneous glioma model was used to evaluate tumour reduction efficacy after treatment with Fc-diOH and Fc-diAc-LNCs. After tumours had developed to about 100 mm³, we performed comparative efficacy studies by dividing animals into four groups according to the treatment they received. As already shown (Allard et al., 2008b), the progression of tumour volume for the rats of control groups increase by a factor 9–10 (Fig. 3). Indeed, tumour volume increased from 240 ± 60 to 1900 ± 1420 mm³ between Day 10 and Day 30 for the animals treated with a single injection of blank LNCs and from 250 ± 100 to 2500 ± 1830 mm³ for the group of rats treated with physiological saline. On the contrary, tumour volume progression for 1 mg/g Fc-diOH-loaded LNCs was significantly reduced ($p < 0.05$). Tumour volume increased from 160 ± 90 to 765 ± 690 mm³ between Day 10 and Day 30 which means that the volume progression in 20 days increased by a factor of 4.8. The results show that a single administration of Fc-diAc-loaded LNCs reduced the tumour volume progression from 235 ± 45 to 700 ± 700 mm³ (factor of 3). The high values of standard deviation show us that the effect of tumour reduction between Fc-diOH-LNCs and its acetyl prodrug were not different ($p = 0.854$). On the contrary, the effect of tumour reduction became significant compared to the two control groups ($p < 0.05$). Nevertheless, in our previous study, we hypothesised that the protection of the phenol functions of Fc-diOH by grafting an acetate chain should allow prolonged activity of Fc-diOH-loaded LNCs. This was not the case. Moreover, formulations of Fc-diAc-LNCs with higher drug loadings (≥ 7.8 mg/g) gave disappointing results as formulations suffered from instability (data not shown). As the results of ectopic models were similar between Fc-diOH and its prodrug Fc-diAc and because

highly concentrated formulations were not stable, dose effect experiments in the orthotopic model were performed only with Fc-diOH, encapsulated in LNCs or solubilised in Labrafac®.

Fig. 3. *In vivo* effects of Fc-diOH and Fc-diAc-loaded LNC treatment on the growth of 9L-glioma cells implanted subcutaneously on Fisher rats. Efficacy of the treatments with ferrocifen-loaded LNCs was compared with controls made after a single injection of physiological serum or blank LNCs. The graph represents an estimation of tumour growth assessed by tumour size measurements $\pm$ SD. Tumours were measured four times a month with callipers and tumour volume was approximated as an ellipsoid. Statistical analysis by pairs shows significant differences on Day 30 for Fc-diOH LNC treatment compared to both control injections. * $P < 0.05$ —Student *t*-test.

3.4. Orthotopic model—Effect of the dose and formulation of Fc-diOH (LNCs or Labrafac®)

In this work, we tested ‘ferrocifen’ antitumoural efficacy on a glioma brain model. Results obtained *in vitro* or in subcutaneous models for malignant glioma have not been shown to be very valuable for predicting therapeutic efficacy (Lamfers et al., 2007) because of the specificity of the brain which is isolated from the rest of the body due to the presence of the blood brain barrier (BBB). Moreover, glioma brain tumours are known to be hypoxic tumours (Bernsen et al., 2000, Khan et al., 2009). Due to this, Fc-diOH was administered into the brain according to two different intracerebral techniques; by simple stereotaxy or by convection-enhanced delivery (CED). CED was introduced in the early nineties as a technique to enhance drug distribution, especially effective when compared to local delivery methods such as stereotaxy based on diffusion (Bobo et al., 1994, Morrison et al., 1994). For a dose-effect study, Fc-diOH was administered encapsulated in LNCs at a drug loading of 1 mg/g and in a concentrated dose of 6.5 mg/g as previously described (Allard et al., 2008b). Furthermore, in order to maximise the dose, Fc-diOH was also infused at its solubility limit in a Labrafac® solution (40 mg/g) which usually constitutes the core of LNCs. These three groups of rats were firstly compared to a control group of rats without brain infusion but that underwent the same anaesthesia. All non-treated rats died within 27 days with a median and mean survival

of 25 days (Fig. 4A; Table 2). There was no increase in life expectancy for the rats treated by Fc-diOH-LNCs 1 mg/g (0.005 µg/rat) stereotaxy as this group had a median and mean survival time of 24 and 24.1 ± 2.1 days respectively ($p = 0.63$). Rats treated with a CED injection of Fc-diOH-LNCs 6.5 mg/g (0.36 mg/rat) showed an increased median survival time of 8% when compared to controls with a median and a mean survival time equivalent to 27 and 28.1 ± 3.1 days respectively. The difference between these two groups appeared to be significant ($p = 0.0098$). Moreover, the experiments established that median survival increased for the group treated with Fc-diOH in solution which corresponded to the highest dose tested (2.5 mg/rat). Median survival time was about 31 days with an increase survival time of 24% compared to the control group. The difference also appeared to be significant compared to the control group ($p = 0.012$). In addition, 1 rat in the Fc-diOH-Labrafac[®] group (14.2%) was a long-term survivor which enhanced the mean increased survival time (IST) up to 58.4% compared to the control (Table 2). These results confirmed the cytostatic activity of Fc-diOH *in vivo* in an orthotopic model and the existence of a dose effect with this drug by brain administration. Moreover, when the groups treated with the diphenol ferrocenyl molecule encapsulated in LNCs were compared to their corresponding control groups i.e. by blank LNCs infused by stereotaxy or by CED, the conclusions were unchanged (Fig. 4B). Therefore, the local delivery of blank LNCs, whatever the infusion method used, was not more toxic than an absence of infusion and gave median survival times similar to control groups ($p > 0.05$). Consequently, the difference in survival time between a CED injection of Fc-diOH-LNCs 6.5 mg/g and blank LNCs was still significant ($p = 0.0338$). On the contrary, the rats treated with Labrafac[®] suffered from lethargy, rapid loss of weight and had a median survival time significantly different than the control group, equivalent to 21.5 days ($p < 0.001$, Fig. 4C). Consequently, the difference in survival time between the group Fc-diOH-Labrafac[®] and the group Labrafac[®] was highly significant with a p value inferior to 0.001. This experiment showed that increasing the dose of Fc-diOH from 0.005 to 2.5 mg/rat allowed an increase of the median from 24 to 31 days, but underlined the problem of the clinical tolerance of Labrafac[®] infused alone, whereas LNCs appeared well-tolerated by the animals.

Fig. 4. Kaplan–Meier survival curves for 9L-glioma bearing rats for escalating doses of Fc-diOH encapsulated in LNCs or solubilised in Labrafac® versus appropriate controls. (A) represents the survival curves in days after tumour implantation for untreated animals = control group (■), stereotaxy of Fc-diOH-LNCs 0.005 mg/rat (○), CED of Fc-diOH-LNCs 0.36 mg/rat (▼), and CED of Fc-diOH-Labrafac® 2.5 mg/rat (○). No significant differences were found between treatment with Fc-diOH-LNCs 0.005 mg/rat and 0.36 mg/rat versus blank LNCs administered in stereotaxy or by CED (B). The difference became significant only for the group treated with the highest dose (Fc-diOH-Labrafac® 2.5 mg/rat) compared to the control group ($p = 0.018$) and also to the group treated with Labrafac® alone ($p < 0.0001$) (C).

Table 2. Descriptive and statistical data from the survival study with Fc-diOH chemotherapy administered by stereotaxy (stereo) ($V = 10 \mu\text{L}$) or by CED ($V = 60 \mu\text{L}$) for escalating doses from 0.005 to 2.5 mg/rat.

Treatment	Injection	<i>n</i>	Survival time (days)			Increase survival time (%)		
			Range	Median	Mean $\pm$ SD	Long-term survivors	IST median	IST mean
Fc-diOH-LNCs 0.005 mg/rat	Stereo	7	21–27	24.0	24.1 ± 2.1	0	0	0
Fc-diOH-LNCs 0.36 mg/rat	CED	8	24–32	27.0	28.1 ± 3.1	0	8	12.4
Fc-diOH-Labrafac [®] 2.5 mg/rat	CED	7	24–100	31.0	39.6 ± 27.1	14.2	24	58.4
Blank LNCs	Stereo	7	21–26	24.0	24 ± 1.6	0	0	0
Blank LNCs	CED	8	21–30	25.0	24.6 ± 2.6	0	0	0
Labrafac [®]	CED	8	21–23	21.5	21.5 ± 0.9	0	0	0
No treatment	CED	9	23–27	25.0	25.0 ± 1.2	0	–	–

n is the number of animals per group. The increases in median and mean survival time (IST_{median} and IST_{mean}) are calculated in comparison to the control group (%).

Work on the biocompatibility of these systems is in progress. Finally, the possibility to obtain freeze-dried LNCs by reducing the final volume of water (Dulieu and Bazile, 2005) could be a good way to obtain higher doses or amount at least equivalent to those obtained for Labrafac[®] infusion (i.e. 2.5 mg/g), without toxicity. These results highlight the cytostatic activity of Fc-diOH in an orthotopic glioma model. This is an important result in the field of bioorganometallic chemistry as far as the bioavailability of organic polyphenols is concerned, this subject having been widely discussed in the literature (Manach et al., 2004, Sang et al., 2005, Williamson and Manach, 2005).

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