Pharmaceutical Research, Vol. 27, No. 1, January 2010 (© 2009)
DOI: 10.1007/s11095-009-0006-0

Research Paper

Local Delivery of Ferrociphenol Lipid Nanocapsules Followed by External Radiotherapy as a Synergistic Treatment Against Intracranial 9L Glioma Xenograft

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Received July 24, 2009; accepted October 27, 2009; published online November 12, 2009

Purpose. The goal of the present study was to evaluate the efficacy of a new organometallic drug, ferrociphenol (Fc-diOH), in combination with external radiotherapy in intracerebral 9L glioma model. We tested the hypothesis that the combination of external radiotherapy with Fc-diOH could potentiate the action of this drug.
Methods. 9L cells were treated with Fc-diOH-LNCs (from 0.01 to 1 μmol/L) and irradiated with external radiotherapy (from 2 to 40 Gy). *In vivo* assessment was evaluated by the inoculation of 9L cells in Fisher rats. Chemotherapy with Fc-diOH-LNCs (0.36 mg/rat) was administered by means of convection-enhanced delivery (CED), and the treatment was followed by three irradiations of 6 Gy doses (total dose=18 Gy).
Results. *In vitro* evaluations evidenced that a combined treatment with Fc-diOH-LNCs and irradiations showed synergistic antitumor activity on 9L cells. Combining cerebral irradiation with CED of Fc-diOH-LNCs led to a significantly longer survival and the existence of long-term survivors compared to Fc-diOH-LNCs-treated animals ($p<0.0001$) and to the group treated with blank LNCs+radiotherapy ($p=0.0079$).
Conclusion. The synergistic effect between ferrociphenol-loaded LNCs and radiotherapy was due to a closely oxidative relationship. Upon these considerations, Fc-diOH-LNCs appear to be an efficient radiosensitive anticancer drug delivery system.

KEY WORDS: convection-enhanced delivery; glioma; iron; lipid nanocapsules; radiotherapy.

INTRODUCTION

Even with aggressive multi-modality treatment strategies, the life expectancy of patients with glioblastoma multiforme (GBM), the most aggressive primary brain tumor, is only slightly longer than 1 year after diagnosis. Surgery and radiotherapy with concomitant and adjuvant temozolomide, a novel oral alkylating agent, is the standard therapy regimen for newly diagnosed GBM. Patients treated with radiotherapy plus temozolomide show a median survival time of 14.6 months *versus* 12.1 months with radiotherapy alone after surgery (1). This moderate result underlines the critical role of chemotherapy, the utility of which is very often controversial (2). In an attempt to overcome the limitations of systemic delivery of anticancer drugs aimed at brain targeting, especially because of the presence of the blood brain barrier (BBB), several methods of regional delivery have been developed (3,4). Convection-enhanced delivery (CED) was introduced in the early nineties as a technique to enhance drug distribution, especially compared to local delivery methods based on diffusion (5,6). Many anticancer drugs have been investigated in CED (7,8), but many problems relating to local CNS toxicity, short drug tissue retention and heterogeneous distribution within tumors were reported. To circumvent these problems, some drugs have been encapsulated in nanocarriers and infused by CED. Nano-encapsulation offers many advantages, such as protection of the active species, reducing both the interaction with the brain extra-cellular matrix (ECM) (9) and brain toxicity (10), a better drug distribution (11) and a longer brain half-life (12,13). In this context, our group focused on the administration and study of the infusion of lipid nanocapsules (LNCs) in rat brain by CED. These lipid nanocarriers presented ideal characteristics for CED infusion, such as a low particle size < 100 nm, and a global negative charge, and they are shielded by a polyethylene glycol (PEG) steric coating (14). Moreover, LNCs can be loaded with many lipophilic drugs, as well as amphiphilic molecules, and appear like a new platform for nanomedicine (15). In the field of GBM therapy, hydrophobic bioorganometallic compounds were encapsulated in LNCs and investigated as a new class of active molecules (16). In this context, we deal with a dual innovation. Bioorganometallic molecules are defined as active molecules that contain at least one carbon directly bound to a

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metal or metalloid. The metal studied here is iron (Fe), and the metallocene derivative is ferrocene [η^5-Fe(C_5H_5)$_2$] chemically grafted on a polyphenolic skeleton. The resulting molecules are ferrocenyl phenol compounds that we called "ferrocifen." They were first studied on breast cancer cell lines, where they showed dual effects: an antiestrogenic effect on estrogen receptor positive cell lines and a cytotoxic effect on hormone independent cell lines (17,18). These compounds are thought to be susceptible to an oxidation of the ferrocenyl antenna to give intracellular quinone methides that are cytotoxic via interaction with the nucleophiles present in the cell (19). We previously demonstrated that LNCs could be loaded with the ferrocenyl diphenol molecule called "ferrociphenol" (Fc-diOH) with high drug-loading levels and that LNCs were an ideal cargo to solubilise and infuse this drug, which is totally insoluble in water. Moreover, lipid nanocapsules exhibited great advantages compared to swollen micelles, produced by a similar process and composed of higher quantities of surfactant (16). *In vitro*, Fc-diOH-LNCs were cytotoxic on 9L glioma cells (IC_{50}=0.6μM). Promising *in vivo* results were also obtained after intratumoral administration of this new drug carrier in a subcutaneous injected 9L glioma model, as it dramatically reduced the tumor mass and glioma volume. Moreover, the cytostatic activity of Fc-diOH-LNCs was confirmed on an orthotopic glioma model but was very modest (20). In the present study, we first evaluated the activity of Fc-diOH-LNCs associated with radiotherapy, on 9L cells in culture. Then, a combined treatment using CED of Fc-diOH-LNCs with external beam radiotherapy was evaluated on 9L glioma-bearing rats.

MATERIALS AND METHODS

Materials

Ferrocenyl diphenol compound (2-ferrocenyl-1,1-bis(4-hydroxyphenyl)-but-1-ene) named Fc-diOH was prepared by a McMurry coupling reaction (21). 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 a gift from Lipoïd Gmbh (Ludwigshafen, Germany) and BASF (Ludwigshafen, Germany), respectively.

Preparation of Fc-diOH-LNCs

Lipid nanocapsules were prepared according to our previously described procedure (16). 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 85°C and 60°C were then carried out and followed by a dilution with 2°C deionised water added to the mixture at 70–75°C. To formulate Fc-diOH-LNCs, a first step consisted in dissolving the anticancer drug in triglycerides (Labrafac®) using ultrasound during 1.5 h. Two parameters were varied: the amount of Fc-diOH in triglycerides (1.7% and 4% (w/w)) and the volume of cold water for LNC dilution (70% and 28.5% v/v) to obtain drug loadings of 1 mg/g (0.84% w/w dry weight) and 6.5 mg/g (2% w/w dry weight), respectively. For *in vivo* applications combined with radiotherapy, sucrose (20% w/w) was dissolved in the aqueous phase of the LNC suspension after formulation.

LNC Physicochemical Properties

LNCs were analyzed for their size and charge distribution using a Malvern Zetasizer® Nano Series DTS 1060 (Malvern Instruments S.A., Worcestershire, UK). LNCs were diluted 1:60 (v/v) in deionised water in order to ensure a convenient scattered intensity on the detector. The viscosities of the suspensions were measured at room temperature using Schott Geräte AVS 400 Model automatic viscometer (Oswald viscosimeter). Each value was reported as an average of five measurements±standard deviation.

Tumor Cell Line

Rat 9L gliosarcoma cells were obtained from the European Collection of Cell Culture (Salisbury, UK, N°94110705). The cells were grown 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).

Irradiation

Radiotherapy was conducted with a linear accelerator (Clinac®, Varian Medical Systems, Salt Lake City, USA). The irradiations were delivered by one beam with energy of 6 MV and with an adapted field according to the material irradiated. For *in vitro* irradiations, cells were irradiated at room temperature as a single exposure to doses of photon of 2, 5, 6, 10, 20, and 40 Gy or in 3 fractions of 6 Gy spaced in time. For animal irradiations, fractionated radiotherapy consisted of 18 Gy given in 3 fractions of 6 Gy over 2 weeks, on Day 8, 11 and 14. The dose rate for the irradiation was 4 Gy/min and four rats were irradiated at a time. The animals were anesthetized before irradiation under light sedation (isoflurane/oxygen anaesthesia 3%/3l min^{-1}) and placed on the Clinac® couch in prone position with laser alignment.

Cell Protocol

The 9L cells were plated on 24-well plates in DMEM containing 10% FCS and 1% antibiotic/antimycotic solutions and then treated with increasing concentrations of Fc-diOH-LNC chemotherapy (CT) and external radiotherapy (RT) according to three different protocols described below.

Protocol RT+CT *versus* CT+RT

The 9L cells were plated on 24-well plates for 24 h at 40,000 cells/well. At Day 1, cells were irradiated with 5 to 40 Gy or treated with Fc-diOH-LNCs 1μmol/L. At Day 2, irradiated cells at Day 1 were treated with Fc-diOH-LNCs 1μmol/L (protocol 1), and cells treated with CT were irradiated with 5 to 40 Gy (protocol 2). MTT survival test was then performed 96 h after (Day 6).

Combined Effect Protocol

9L cells were plated at 3,000 cells/well at Day 0. At Day 2, they were treated with increasing concentrations of Fc-diOH-LNCs from 0.01 to 1 µmol/L. At Day 3, cells were irradiated with 2, 6, 10, 20 or 40 Gy, and MTT was performed 96h after (Day 7). The synergy of the combined treatment was assessed using isobologram analysis (22). To that, the IC_{50} values, i.e. the Fc-diOH concentrations and the irradiation dose at which 50% of the 9L cells survived, were determined. To establish the IC_{50} values for CT alone, cells were incubated with increasing concentrations of Fc-diOH from 0.001 to 100 µmol/L. For the evaluation of the IC_{50} in RT alone, cells were irradiated at 5, 10, 20 and 40 Gy in a single fraction.

Multi-Irradiation Protocol

9L cells were plated at 500 cells/well at Day 0, treated with increasing concentrations of Fc-diOH-LNCs from 0.01 to 1 µmol/L at Day 4 and irradiated with 3 fractions of 6 Gy at Day 5, 8 and 11. MTT survival test was performed at Day 15.

MTT Survival Test

After 96 h within any treatment, cell survival percentage was estimated by the MTT survival test. 40 µl of MTT solution at 5 mg/ml in PBS were 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 ($OD_{control}$). Cell survival percentage was estimated according to Eq. (1). Each experiment was conducted two times with at least six repeated samples.

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

Animals and Intracranial Xenograft Technique

Syngeneic Fischer F344 female rats weighing 160–180 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 anesthetized with an intraperitoneal injection of 0.75–1.5 ml/kg of a solution containing 2/3 ketamine (100 mg/ml) (Clorketam®, Vétoquinol, Lure, France) and 1/3 xylazine (20 mg/ml) (Rompun®, Bayer, Puteaux, France). Animal care was carried out in strict accordance with French Ministry of Agriculture regulations. A 9L tumor monolayer was detached with trypsin-ethylenediamine tetraacetic acid, washed twice with EMEM (Eagle's Minimal Essential Medium) without FCS or antibiotics, counted and resuspended to the final concentration desired. For intracranial implantation, 10 microliters of 1,000 9L cell suspension were injected into the rat striatum at a flow rate of 2 µl per minute using a 10 µl syringe (Hamilton® glass syringe 700 series RN) with a 32G needle (Hamilton®). For that purpose, rats were immobilized 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 per minute).

Convection-Enhanced Delivery

On Day 6, 60 µl of the LNC suspensions were injected by CED at the coordinates of the tumor cells. Infusions were performed at the depth of 5 mm from the brain surface using a 10 µl Hamilton® syringe with a 32G needle. This syringe was connected to a 100 µl Hamilton syringe 22G containing the product (Harvard Apparatus, Les Ulis, France) through a cannula (CoExTM PE/PVC tubing, Harvard apparatus, Les Ulis, France). CED was performed with an osmotic pump PHD 2,000 infusion (Harvard Apparatus, Les Ulis, France) by controlling a 0.5 µl/min rate for 2 h. Sixty-two rats with 9L tumor cells were randomized into five experimental groups. The groups were as follows: (1) control group without CED but with the same anesthetized scheme (n=9); (2) CED group of blank LNCs, (3) CED group receiving CED of Fc-diOH-LNCs at a dose of 0.36 mg/rat (n=8); (4) radiotherapy group receiving CED of blank LNCs followed by whole-brain radiation to a total dose of 18 Gy (3x 6 Gy) (n=10), (5) CED plus radiotherapy group receiving CED of Fc-diOH-LNCs at a dose of 0.36 mg/rat followed by whole-brain radiation to a total dose of 18 Gy (n=19). For the rats treated with radiotherapy (with or without chemotherapy), sucrose was added to the formulations.

Statistical Analysis

Data from *in vitro* experiments are presented as mean±SD, and statistical analysis between groups was conducted with the two-tailed Student t-test ($p<0.05$ was considered to be signifi-

Table I. Physicochemical characteristics of blank and Fc-diOH-LNCs

	Mean particle size (nm)	Polydispersity index (PDI)	Zeta potential (mV)	Viscosity ($mm^2.\ s^{-1}$)
Blank LNCs	48.7±0.5	0.063±0.012	−9.4±0.2	4.4±0.1
Blank LNCs+sucrose	47.6±0.1	0.048±0.009	−9.5±1.1	8.7±0.2
Fc-diOH LNCs 1 mg/g	46.3±0.7	0.050±0.009	−9.6±3.9	–
Fc-diOH LNCs 6.5 mg/g	45.3±2.5	0.074±0.039	−10.5±1.0	–
Fc-diOH LNCs 6.5 mg/g+sucrose	46.6±2.1	0.103±0.074	−10.0±1.1	–

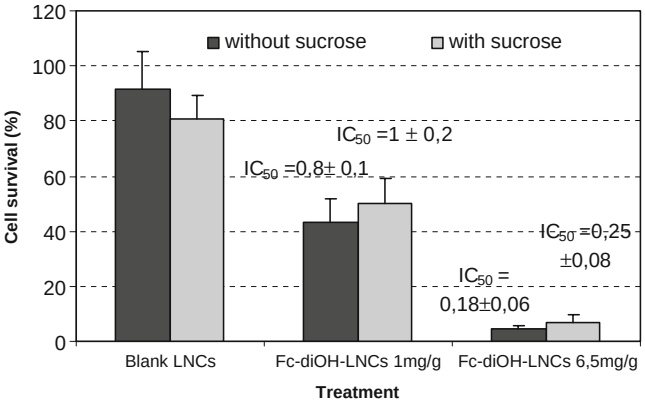

Fig. 1. Cell survival test after treatment with Fc-diOH-loaded and blank LNCs with or without sucrose in external phase. Formulations were diluted in culture medium with the same dilution factor (1/2.350). Final concentrations of Fc-diOH were equivalent to 0, 1 and 6.5 µmol/L for Blank LNCs, Fc-diOH-LNCs 1 mg/g and Fc-diOH-LNCs 6.5 mg/g, respectively. IC50 values were about 0.8 to 1 µM for Fc-diOH-LNCs 1 mg/g and about 0.18 to 0.25µM for Fc-diOH-LNCs 6.5 mg/g without and with sucrose, respectively.

cant). 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 median survival time (days), increase in survival time (IST_{median} %), and long-term survivors (%).

RESULTS

LNC Formulation and Cytotoxicity

As shown in previous work (16), Fc-diOH-LNCs presented a very narrow size between 45.3 and 48.7 nm, depending on the drug payload, and were monodispersed (PDI ≤ 0.1) (Table I). In opposition to what was observed with more lipophilic drugs containing acetyl or palmitoyl chains as protecting groups (20), neither recrystallisation nor phase separation was observed. The presence of sucrose in the LNC suspension aqueous phase did not affect the size, as there was no significant change in size measurements. Zeta potential values were equivalent from −9.4 to −10.5 mV for all the suspensions. On the contrary, viscosity increased from 4.4±0.1 to 8.7±0.2 mm^2/s with the presence of the disaccharide in the formulation. The presence of sucrose had no toxic effect on cells *in vitro*, as there was no significant difference in cytotoxicity between blank and Fc-diOH-LNCs with or without sucrose (Fig. 1). Moreover, a dose effect for Fc-diOH could be observed after its encapsulation, as 9L cell survival was of 45–50% for 1 mg/g strength Fc-diOH-LNCs and 4–6% for 6.5 mg/g loaded nanocapsules.

Combined Effects of Chemotherapy and Radiotherapy

Two distinct protocols combining chemotherapy and radiotherapy were performed on 9L cells. One group of cells was treated with radiotherapy followed by chemotherapy with Fc-diOH-LNCs (protocol 1=RT+CT), and another group received the chemotherapy before the RT regimen

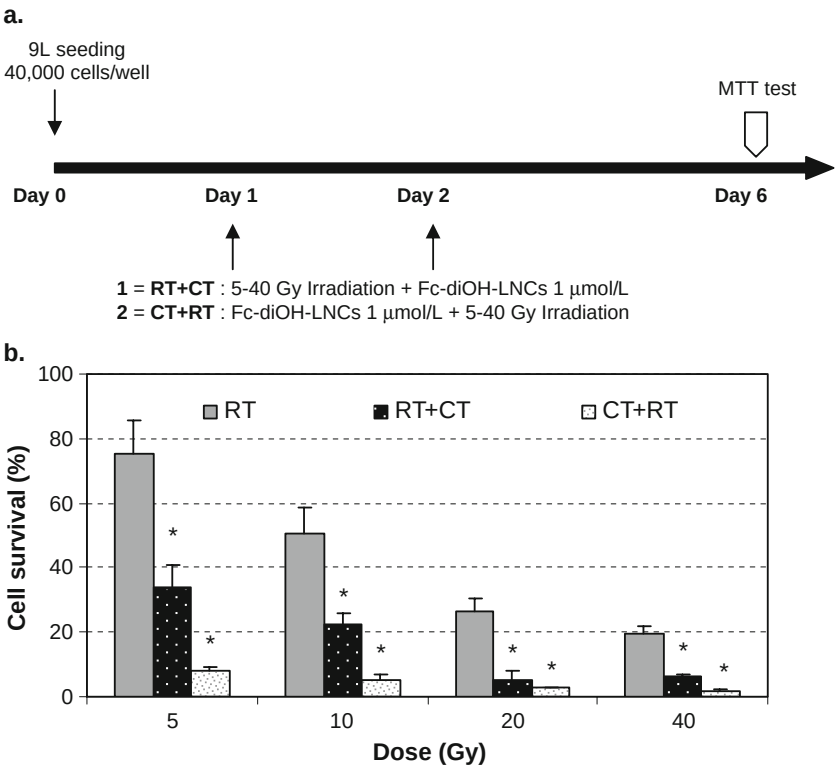

Fig. 2. Cell survival percentage of 9L cells according to two distinct protocols: radiotherapy+chemotherapy (RT+CT: protocol 1) *versus* chemotherapy+radiotherapy (CT+RT: protocol 2). Chemotherapy was a treatment with Fc-diOH-LNCs 1 mg/g at 1 µmol/L, and Radiotherapy was a single irradiation at 5, 10, 20 and 40 Gy (* means $p<0.05$, Student's *t* test).

(protocol 2=CT+RT) (Fig. 2a). First, cell survival percentage decreased with the increasing dose of radiotherapy for all conditions tested (Fig. 2b). For the first protocol tested (RT+CT), the percentage of cell survival decreased from 34% to 6% for 5 and 40 Gy, respectively. For protocol 2, CT+RT, cell survival percentages decreased from 8% to 2% for the cells irradiated between 5 and 40 Gy, respectively. The difference in cell viability was shown to be significant between radiotherapy alone and protocol 1 but was also significant for protocol 1, RT+CT, *versus* protocol 2, CT+RT, ($p<0.05$). As the treatment was more efficient when cells were first treated by chemotherapy, the sequential utilization of Fc-diOH treatment followed by RT was selected for the following studies.

Synergistic Effects

To determine if the chemotherapy-radiotherapy association with Fc-diOH and external beam photon irradiation was an additive or a synergistic effect, isobologram analysis was performed. Cells were plated at Day 0, treated with increasing concentrations of Fc-diOH-LNCs at Day 2 and irradiated 24 h after by 2, 6, 10, 20 and 40 Gy (Fig. 3a). Median effect doses (IC_{50} values) calculated from this experiment were 0.4 µmol/L for Fc-diOH-LNCs and about 7.5 Gy for irradiation therapy (Fig. 3b–c). The combination of Fc-diOH treatment with radiotherapy of 2 and 6 Gy allowed the determination of IC_{50} values whereas irradiations with 10, 20 and 40 Gy without chemotherapy always gave cell survival percentage less than 50% (Fig. 3b). The IC_{50} values were about 0.15 µmol/L and 0.01 µmol/L for 2 Gy and 6 Gy, respectively. As these values, divided by the IC_{50} were under the dash line of the isobologram (Fig. 3d), this result indicated a synergistic effect between the two treatments.

Multi-Irradiation

Cultured monolayers of 9L cells were treated with increasing concentrations of Fc-diOH-LNCs followed by three irradiations doses leading to a final dose of 6, 12 and 18 Gy (Fig. 4a). Cell survival percentage was calculated after performing the MTT survival test (Fig. 4b). These results

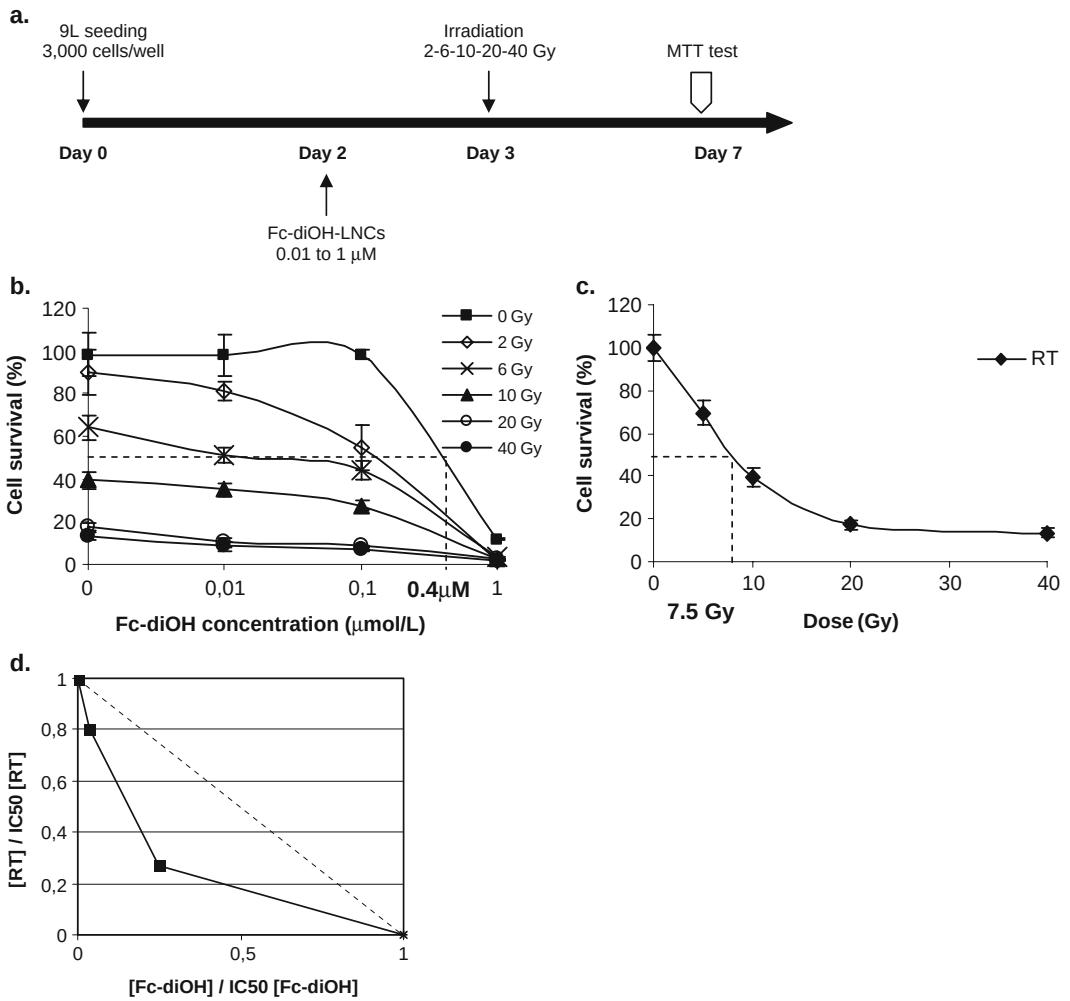

Fig. 3. Synergistic effect of cell death by Fc-diOH-LNCs and radiotherapy in 9L glioma cells. Cells were treated according to the protocol detailed in (**a**). Cell survival percentage of 9L cells treated with CT (from 0.01 to 1 µM)+ RT (from 2 to 40 Gy) were expressed in (**b**). IC_{50} for CT alone (RT=0Gy) was equivalent to 0.4 µM (**b**) and IC_{50} for RT (CT=0µM) was about 7.5 Gy (**c**). Synergy was determined between the two treatments as seen in the isobologram analysis (points below dotted line) (**d**).

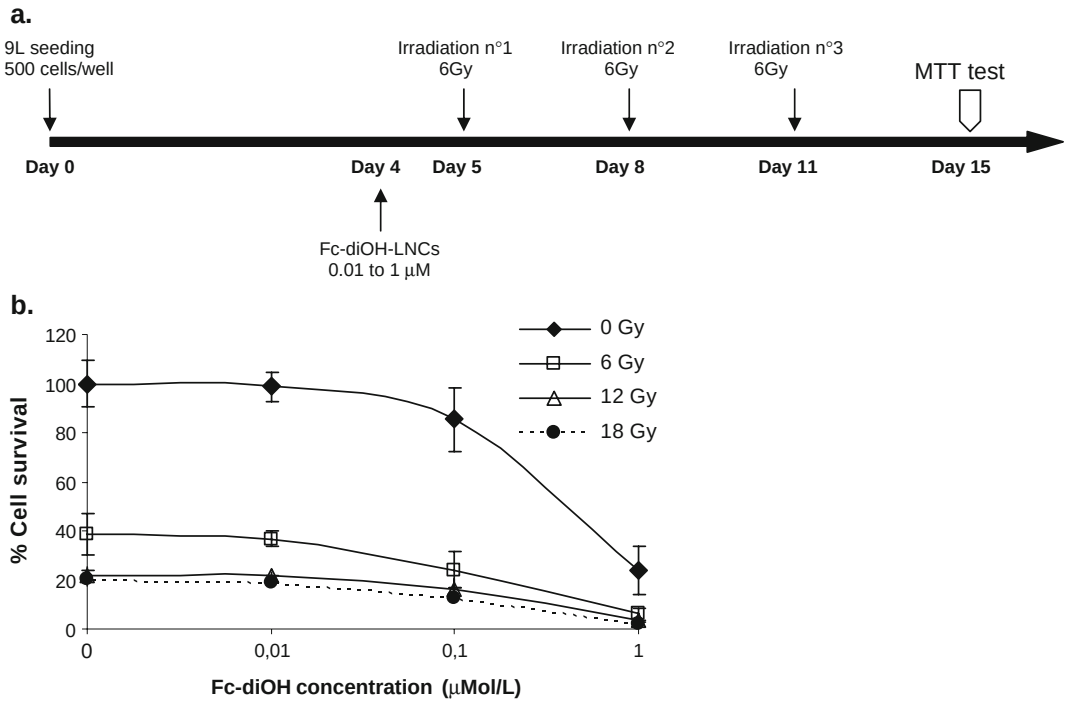

Fig. 4. 9L cell viability after a chemotherapy treatment with Fc-diOH-LNCs followed by a multi-irradiation scheme of 3x6 Gy. Multi-irradiation protocol is detailed in (**a**). (**b**) represents the cell survival percentage after treatment with Fc-diOH-loaded LNCs at concentrations between 0.01 and 1 µM and followed by a total dose of RT of 0, 6, 12 and 18 Gy (MTT assay).

showed that cell death is directly proportional to Fc-diOH concentration. Moreover, cell survival percentage decreased with the repetitive irradiations as 23.9%, 6.1%, 3.2% and 1.9% of the cells were still alive after a treatment with 1 µM Fc-diOH-LNCs, followed by 0, 1, 2 and 3 irradiations, respectively (Fig. 4b). By calculation, the effect of cell death was shown to be predominant after the first irradiation, whatever the chemotherapy scheme. For a chemotherapy treatment without radiotherapy, cell death percentage increased up to 76.1% for a single treatment with Fc-diOH-LNCs 1 µmol/L. The percentages of cell death spread from 61.4 to 74.4% between 0 and 6 Gy, decreased from 33.0% to 48.4% for the second irradiation (6–12 Gy) and finished between 5.3% to 38.2% for the last irradiation (12–18 Gy). After the third irradiation, the cell death percentage was much higher for the cells treated with the highest dose of Fc-diOH, especially compared to the group of cells only treated with radiotherapy (38.2% *versus* 5.3%).

Survival Study

9L tumor-bearing rats were treated either by a CED injection of 6.5 mg/g strength Fc-diOH-LNCs (0.36 mg/rat), a CED injection of unloaded LNCs, a CED injection of Blank LNCs followed by irradiation with 3 fractions of 6 Gy over 7 days (total dose=18 Gy) or a CED injection with 6.5 mg/g strength Fc-diOH-LNCs (0.36 mg/rat) followed by local irradiation (Fig. 5a). A control group without CED injection but undergoing the same anesthetized scheme was also preformed. All non-treated rats died within 27 days with a median survival of 25 days (Fig. 5b and Table II). Among them, the injection of the unloaded carrier gave a median survival time of 25 days, and all the animals died within 30 days. As shown in previous study, there was a slight increase in life time for the rats that were only treated with chemotherapy (20). Rats treated with a CED injection of blank LNCs followed by 18Gy irradiation showed an increased median survival time of 32% when compared to controls. The result in survival time was significantly different compared to the control group ($p<0.0001$). Combination of Fc-diOH and RT further improved survival as the median survival time was equivalent to 40 days. The experiments established that rat median survival was improved significantly for this group compared to control groups and to chemotherapy group ($p<0.0001$) but also compared to the group treated with blank LNCs+RT ($p=0.0079$). In addition, 2 rats (10.5%) in the Fc-diOH+RT group were long-term survivors (Table II).

DISCUSSION

Polyphenols have a variety of biological activities, ranging from anti-aging or anticancer activities to lowering blood cholesterol levels and improving bone strength (23,24). Among synthetic phenol compounds, ferrocenyl diphenol structures have been particularly studied as anticancer agents (25). The incorporation of the organometallic group ferrocene in small organic phenols was performed to enhance the cytotoxicity of this type of molecule (21). Within all these molecules called "ferrocifens," the most active examples contained the 2-ferrocenyl-1-phenyl-but-1-ene motif (26), and the representative of this class was the 2-ferrocenyl-1,1-bis(4-hydroxyphenyl)-but-1-ene compound called ferrociphenol (Fc-diOH). Nevertheless, this ferrociphenol compound is not sufficiently soluble in water to allow its direct

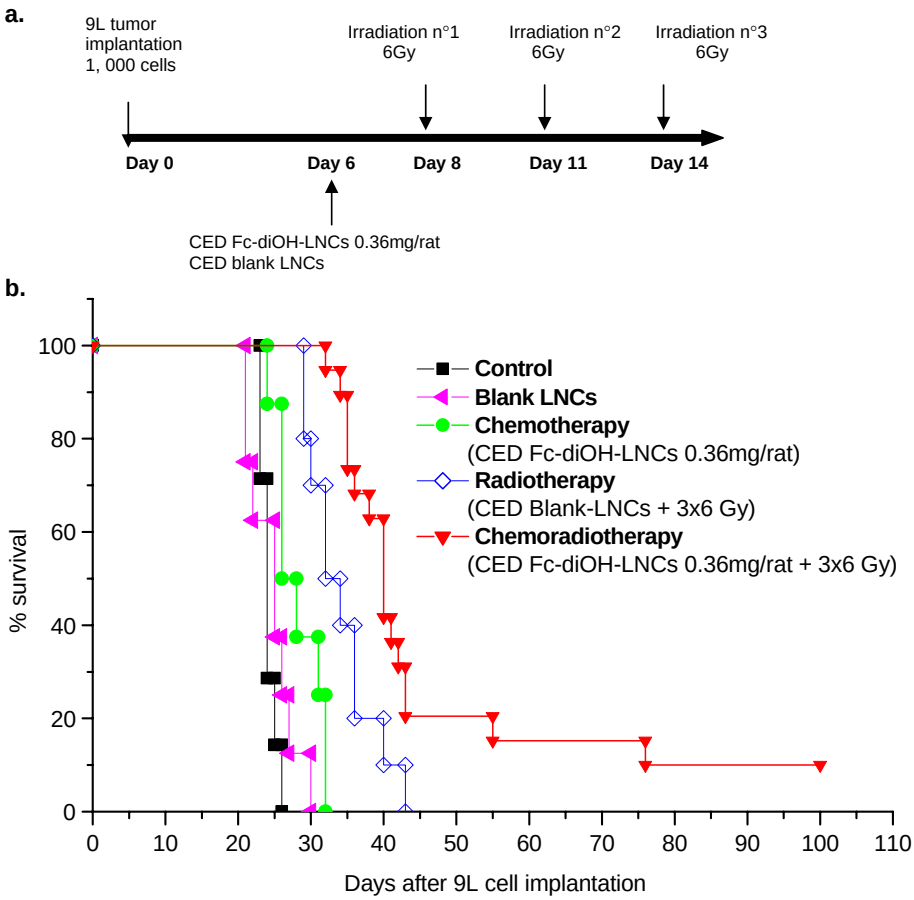

Fig. 5. Representation of the chemoradiotherapy protocol applied on 9L glioma-bearing rats (**a**). Kaplan-Meier survival curves for 9L glioma-bearing rats after CED of Fc-diOH-LNCs and external radiotherapy 3x6 Gy (**b**). Survival times in days after tumor implantation have been plotted for untreated animals (-■-), CED of blank LNCs (-◀-), CED of Fc-diOH-LNCs 0.36 mg/rat (-●-), irradiation 18 Gy (3 fractions of 6 Gy) in combination with CED of blank LNCs (-◊-), and irradiation 18 Gy in combination with CED of Fc-diOH-LNCs 0.36 mg/rat (-▼-). Fc-diOH was administrated on Day 6, and X-ray dose fractions were delivered on Days 8, 11 and 14 after tumor implantation. * means $p<0.05$.

administration. Recently, the Fc-diOH compound was encapsulated in PEG-PLA nanoparticles (27), in methylated β cyclodextrins (Me-β-CD) (28) and in lipid nanocapsules (16), thus allowing its *in vivo* administration.

In this study, Fc-diOH was encapsulated in LNCs at two different drug loadings and tested first *in vitro* on 9L cell lines. The Fc-diOH encapsulation was optimal, as LNC size and zeta potential values were not affected by the presence of the organometallic molecule. Moreover, a dose effect was evidenced *in vitro* on 9L cell lines, as toxicity was more than 10 times higher for 6.5 mg/g strength Fc-diOH-LNCs *versus* 1 mg/g strength Fc-diOH-LNCs. In a previous study, promising *in vivo* results were obtained after intratumoral administration of this drug carrier in a 9L subcutaneous glioma model, but no dose effect was demonstrated between the two drug loading formulations (16). Many of these drugs—polyphenols in general—show promising results *in vitro* but are characterized by a poor bioavailability in animal models.

Table II. Descriptive and statistical data from the survival study with Fc-diOH chemotherapy and external radiotherapy of 18 Gy (3x6 Gy)

		Survival time(days)		Increase life time(%)	
Treatment	n	Range	Median	Long term survivors	IST median
6.5 mg/g Fc-diOH LNCs	8	24–32	27.0	0	8
Blank LNCs+Radiotherapy	10	29–44	33.0	0	32
6.5 mg/g Fc-diOH-LNCs+Radiotherapy	19	32–100	40.0	2	60
Blank LNCs	8	21–30	25.0	0	–
Control	9	23–27	25.0	0	–

n is the number of animals per group. The increase in median survival time (IST median) is calculated in comparison to the control group (%).

To potentiate the action of the drug, Fc-diOH was associated with external beam irradiation. The radiosensitization effects of Fc-diOH-LNCs were first studied *in vitro* on 9L cell cultures. Notably, a higher toxic dose-enhancement ratio was revealed for the chemotherapy (CT)-radiotherapy (RT) protocol in comparison with the RT-CT procedure. A radiosensitizing effect of Fc-diOH can explain the enhanced efficacy of the combined treatment in our model, as better results were observed when Fc-diOH was administered before radiotherapy. Moreover, the treatment combination CT+RT showed synergy and not only additive effects. Irradiations are known to produce free radicals originating from water radiolysis. In the presence of oxygen, highly oxidising radicals are created which interact with various compounds to form hydrogen peroxide (H_2O_2), a very strong oxidising molecule (Fig. 6). Thus, due to an electron transfer phenomenon, the ferrocene unit can be oxidized to ferrocenium, which after some rearrangements generates a quinone methide (19). This quinone methide, which is an alkylating molecule, may react with GSH, DNA and proteins leading to cell death (Fig. 6).

Fractionated external-beam radiotherapy is the standard treatment for the management of malignant gliomas (29). For a similar total dose, the biological efficiency varies according to the total number of sessions (fractionation), the dose per session and the total duration of treatment. In our study, we investigated a radiotherapy scheme in three fractioned doses of 6 Gy a week, and the impact of this scheme was first studied *in vitro* on 9L cell lines. The results showed that cell toxicity was all the more important as the number of doses increased from one to three, and the profit was better for the schemes associated with Fc-diOH-LNC chemotherapy. For all the conditions tested, the main benefit in cell toxicity was obtained after the first irradiation (60–75% cell death) and was slightly reduced after the second and the third irradiation. But the most important observation is that the synergistic effect between Fc-diOH-LNCs and RT was most visible after the third irradiation, as toxicity increased from 5.3% to 38.2% for the cells treated with RT alone *versus* CT+RT, respectively (Fc-diOH-LNCs with the highest dose tested). After the third irradiation, the percentage of cells still alive was mainly due to a radioresistance mechanism. Indeed, 9L cells are classified as a radioresistant cell line, especially compared to other rodent glioma cell lines. Bencokova *et al.* described a surviving fraction at 2 Gy (SF2) of 71.9% for 9L cells against 53.0% and 41.4% for C6 and F98 cell lines, respectively (30). This radioresistance seems to be connected to a high expression of BRCA1, a protein involved in the repair of damaged DNA.

The main objective of this work was to confirm the results obtained in *vitro* in an intracranial *in vivo* model. For that, Fc-diOH-loaded LNCs with the highest dose entrapped (i.e. 0.36 mg/rat) were administered in 9L glioma-bearing rats by convection-enhanced delivery (CED) and then followed by an external radiotherapy of 18 Gy. The major finding of this work is that Fc-diOH administered by CED in combination with external beam irradiation resulted in a significant enhancement in median survival time compared to the chemotherapy group ($p<0.0001$) and also compared to the rats treated by blank LNCs followed by the same irradiation protocol ($p<0.05$). Radiation therapy is known to be an effective postoperative treatment, as it increases the survival time for patients compared to surgery alone (31,32). In the group of interest, two rats were long-term survivors, as they survived up to 100 days, which certainly involves a total eradication of the tumor.

As shown in previous study, the IST median increased from 8% for the group treated with chemotherapy alone (20). In this study, we showed that the association chemotherapy with Fc-diOH-LNCs and radiotherapy was beneficial because the IST median increased from 60% *versus* 32% for the group only treated with radiotherapy. In the present work and in an attempt to improve the volume of distribution (Vd), sucrose was dissolved in the external phase of the LNC suspension. Thus, the viscosity was increased twofold with the presence of sucrose in the formulation. Sucrose was shown to be non-toxic for 9L cells in culture and was used to enhance the

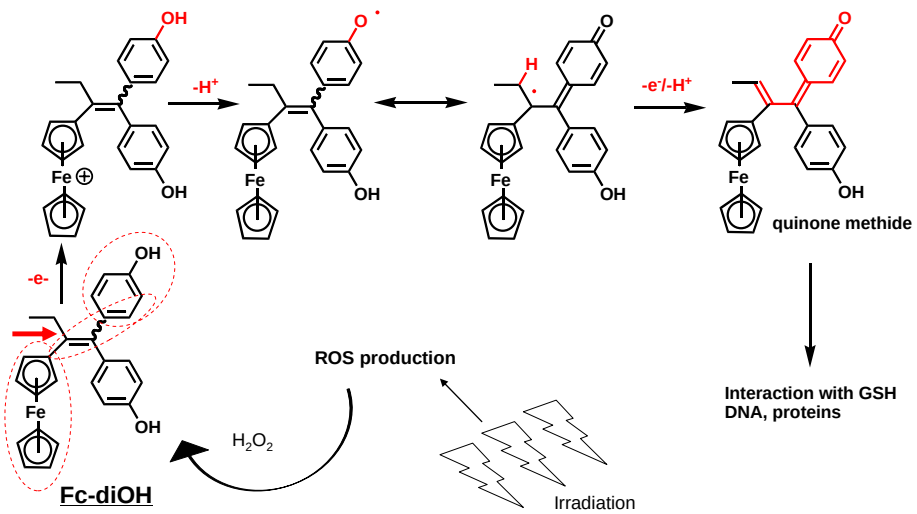

Fig. 6. Proposed mechanism for Fc-diOH cytotoxicity based on electron-transfer studies. Irradiation by the generation of reactive oxygen species (ROS) can form hydrogen peroxide (H_2O_2). H_2O_2, which is a strong oxidizing molecule, can boost the oxidation of ferrocene in ferrocenium, which, after rearrangement, generates a quinone methide strongly cytotoxic. This substance may interact with intracellular sub-units like GSH, DNA or proteins leading to cell death.

viscosity of the infusate. As already described (33,34), high viscosity of the infusate may reduce backflow, thus increasing Vd.

Our data represent the first demonstration of a synergy between these organometallic compounds and an external beam RT, and potentially indicate a therapeutic option for this class of molecules which often suffer from problems of bioavailability.

ACKNOWLEDGMENTS

The authors would like to thank Emilien Porcher, N. Trinh Huynh (Inserm U646, Angers, France), and Pierre Legras (Service Commun d'Animalerie Hospitalo-Universitaire, Angers, France) for skillful technical support with animals, and Elisabeth Hillard for English corrections. This work was supported by a "Région des Pays de la Loire" grant, by the "Cancéropôle Grand Ouest" and by "La Ligue Nationale Contre le Cancer" (équipe labellisée 2007).

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