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Note

Brain tumour targeting strategies via coated ferrociphenol lipid nanocapsules

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ABSTRACT

In this study, a new active targeting strategy to favour ferrociphenol (FcdiOH) internalisation into brain tumour cells was developed by the use of lipid nanocapsules (LNCs) coated with a cell-internalising peptide (NFL-TBS.40–63 peptide) that interacts with tubulin-binding sites. In comparison, OX26 murine monoclonal antibodies (OX26-MAb) targeting transferrin receptors were also inserted onto the LNC surface. The incorporation of OX26 or peptide did not influence the *in vitro* antiproliferative effect of FcdiOH–LNCs on the 9L cells since their IC50 values were found in the same range. *In vivo*, intracerebral administration of OX26–FcdiOH–LNCs or peptide–FcdiOH–LNCs by convection enhanced delivery did not enhance the animal median survival time in comparison with untreated rats (25 days). Interestingly, intra-carotid treatment with peptide–FcdiOH–LNCs led to an ameliorated survival time of treated rats with the presence of animals surviving until days 35, 40 and 44. Such results were not obtained with OX26-MAbs, demonstrating the benefit of NFL-TBS.40–63 peptide as an active ligand for peripheral drug delivery to the brain tumours.

1. Introduction

Nanotechnology has emerged as an interesting field in engineering and designing new systems for drug delivery in oncology such as brain tumour therapy. It can provide medical and pharmaceutical benefits because it enables the control of drug characteristics such as solubility, bioavailability, vascular circulation time, and site-specific delivery [1]. Our laboratory has developed and patented a novel nanoscale system, the so-called lipid nanocapsules (LNCs) [2]. They present an oily core corresponding to medium-chain triglycerides surrounded by tensioactives providing a cohesive membrane made of lecithin and polyethylene glycol 660 hydroxystearate at high density. Thanks to their oily core, these LNCs allow the encapsulation of various lipophilic compounds and represent a potential platform for drug delivery in cancer therapy [2]. Since recently, we have encapsulated into LNCs the organometallic anticancer 2-ferrocenyl-1,1-bis(4-hydroxyphenyl)-but-1-ene (or FcdiOH). These FcdiOH–LNCs were shown to be promising in the 9L rat gliosarcoma model [3]. In order to improve the drug delivery to the site of interest, i.e. the cancer tissue, further modifications of the LNC surface by coating with longer PEG chains in a context of a passive targeting strategy, or by incorporating specific molecules through an active targeting strategy, can be carried out [2]. Two main targeting agents are of particular interest: OX26 murine monoclonal antibody (OX26-MAb) to the rat transferrin receptor (TfR) and NFL-TBS.40-63 peptide derived from the light neurofilament subunit (NFL).

Since the transferrin receptor is overexpressed at the surface of brain tumour cells but is also present in the blood–brain barrier [4], antibodies against the TfR such as OX26 conjugated to nanoparticles have been widely assessed for brain-specific delivery [5,6]. Furthermore, in a previous study, we assessed the targeting ability of the antibody covalently attached onto the LNC surface, and its beneficial effect on drug delivery into a healthy brain has been demonstrated [7].

NFL-TBS.40-63 peptide that specifically interacts with tubulin-binding sites (TBSs) was shown to enter in multiple cancer cell lines leading to disruption of their microtubule network and reduction of their proliferation [8].

Taking those advantages into consideration, we compared in the present study the effect of peptide–FcdiOH–LNCs and OX26–FcdiOH–LNCs on the proliferation of 9L gliosarcoma cells *in vitro* and *in vivo*. In addition, to favour the drug delivery to the site of

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interest, two loco-regional administration routes were assessed, an intra-tumour injection by convection enhanced delivery (CED) and a peripheral injection via the carotid artery.

2. Materials and methods

2.1. Chemical materials

Ferrociphenol was prepared by a McMurry coupling reaction [9]. The lipophilic Labrafac® WL1349 (caprylic-capric acid triglycerides) was purchased from Gattefosse S.A. (Saint-Priest, France). Lipoïd® S75-3 (soybean lecithin at 69% of phosphatidylcholine) came from Lipoïd Gmbh (Ludwigshafen, Germany); Solutol® HS15 (a mixture of free polyethylene glycol 660 and polyethylene glycol 660 hydroxystearate) from BASF (Ludwigshafen, Germany) and NaCl 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). DSPE–mPEG2000–maleimide was kindly provided by Avanti Polar Lipids (Alabaster, USA).

2.2. Tumour cell line and culture

Rat 9L gliosarcoma cells were obtained from the European Collection of Cell Culture (Sigma, Saint-Quentin Fallavier, France). The cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2 in Eagle's minimal essential medium (EMEM) (Lonza, Verviers, Belgium) supplied with 1% non-essential amino acids (Lonza), 10% foetal calf serum (FCS) (Lonza) and 1% antibiotic and antimycotic solution (Sigma).

2.3. Animals

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

2.4. Preparation of the LNCs

2.4.1. Ferrociphenol-loaded LNCs

LNCs were prepared according to the original described procedure [2]. Briefly, the preparation process involved 2 steps. Step I consisted of mixing all the components (Solutol® HS15 (17% w/w), Lipoid® S75-3 (1.5% w/w), Labrafac® WL1349 (20% w/w), NaCl (1.75% w/w) and water (59.75% w/w)) under magnetic stirring and heating from room temperature to 85 °C. Three cycles of progressive cooling and heating between 85 and 60 °C were then carried out. Step II was an irreversible shock induced by sudden dilution with cold water (28.5% (v/v)) to the mixture at 70–72 °C. Slow magnetic stirring was then applied to the suspension for 5 min.

To load the anticancer agent into the oily core of LNCs, FcdiOH was firstly dispersed in Labrafac® under ultrasound at 4% (w/w) for 30 min, and the resulting lipophilic phase was then mixed with other components, as described above, to prepare a suspension of FcdiOH–LNCs at a concentration of 6.5 mg of FcdiOH per g of LNC suspension.

2.4.2. Peptide–FcdiOH–LNCs

Prior to incubation with the peptide (sequence: YSSY-SAPVSSSLSVRRYSSSSGS), the FcdiOH–LNC suspension was passed through a PD-10 sephadex column (Amersham Biosciences Europe, Orsay, France) and then concentrated via centrifugations by using a Millipore Amicon 100 kDa centrifugal filter device (Millipore, St. Quentin-Yvelines, France). The resulting LNC suspension (1 mL) was then incubated for 24 h with 369 μL of 1 mM peptide solution in MiliQ water at room temperature under slow magnetic rotation.

2.4.3. OX26–FcdiOH–LNCs

OX26–FcdiOH–LNCs were prepared following the previously described procedure [7]. Briefly, DSPE–mPEG2000–maleimide was incorporated to the surface of FcdiOH–LNCs 6.5 mg/g at the final concentration of 20 mM by the post-insertion technique at 60 °C for 2 h to formulate functionalized LNCs. The resulting LNC suspension was then passed through a Sepharose CL4-B column, equilibrated with HEPES buffer (pH 7.4, 0.1 M) to separate the functionalized LNCs from micelles composed of free DSPE–PEG2000–maleimide.

OX26 MAb was purified from a supernatant harvested from cultures of the OX26 hybridoma cell line. The obtained OX26 MAb underwent a thiolation process followed by incubation over night at room temperature with functionalized FcdiOH–LNCs leading to the formation of OX26–FcdiOH–LNCs.

2.5. LNC characterisation

The determination of the drug loading is achieved by spectrophotometry at 450 nm after dilution at 1:10 in a 22/67/11 (v/v/v) Ethanol/THF/water mixture.

The average hydrodynamic diameter and the polydispersity index (PI) of nanocapsules were determined at 25 °C, in triplicate, by using a Malvern Zetasizer® (Nano Serie DTS 1060, Malvern Instruments S.A., Worcestershire, UK). For the measurement, the LNCs were diluted at 1:100 (v/v) in deionised water.

2.6. In vitro cell viability

A suspension of 9L cells (1.9×10^4 cells/mL) was put on each well of 24-well plates for 48 h. On day 2, the culture media was removed, and cells were treated with free FcdiOH or different types of FcdiOH–LNC suspensions in the concentration range from 0.01 to 100 μmol/L.

After 72 h of incubation at 37 °C, the media containing treatments was replaced by new media. Cell survival percentage was estimated by the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) survival assay (Promega, Lyon, France). Briefly, the MTS solution (100 μL) was added into each well, and the plates were incubated at 37 °C for 2 h 30. The optical density values (OD) were measured at 490 nm for dark purple intensity and at 750 nm for subtraction of background using a multiwell-scanning spectrophotometer (Multiskan Ascent, Labsystems SA, Cergy-pontoise, France). The maximal absorbance was determined by incubating cells with culture media and was considered as 100% survival. All experiments were performed in triplicate and presented as Mean ± SD.

2.7. In vivo studies

2.7.1. Intracranial gliosarcoma cell inoculation

The animals were anaesthetised by intra-peritoneal injection of 1.0 ml/kg of a 1:1 (v/v) mixture of ketamine (100 mg/ml) (Clorketam®, Vétoquinol, Lure, France) and xylazine (20 mg/ml) (Rompun®, Bayer, Puteaux, France). The incision site was shaved, and the head was immobilised in a stereotaxic frame (Lab Standard Stereotaxic; Stoelting, Chicago, IL). A middle scalp incision was made, and a burr hole was drilled into the skull using a small dental drill. Ten microlitres of 10^5 9L cell/mL suspension was injected stereotaxically into the rat right striatum as previously performed [3] with the following coordinates: 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).

2.7.2. Efficacy study

On day 6 after 9L cell implantation, tumour-bearing rats were treated with OX26–FcdiOH–LNCs or peptide–FcdiOH–LNCs by CED administration (60 μL), or intra-carotid injection (400 μL). The procedures of CED and intra-carotid injection were detailed elsewhere [10]. The untreated control group did not receive any treatment. Animals were weighed every day. Rats were sacrificed in a CO_2 chamber when they lost 20% of body weight and/or as soon as any distress symptom appeared. The death was recorded as if it had occurred on the next day of sacrifice and was represented as the survival time of animals on the Kaplan–Meier curves.

2.7.3. Statistical analysis

Statistical comparisons between the control group and the treated groups were estimated from the log-rank test (Mantel–Cox Test) by using StatView software, version 5.0 (SAS Institute Inc.). The level of significance was set at $P < 0.05$.

3. Results and discussion

3.1. Physicochemical properties of LNC suspensions

Blank LNCs presented an average hydrodynamic diameter of 47.6 ± 0.8 nm and a very narrow size dispersion (PdI = 0.041). As described in a previous study relating the good encapsulation of FcdiOH into the oily core of LNCs [3], its loading did neither alter the particle size nor the PdI (45.0 ± 2.5 nm and 0.054, respectively). The adsorption of NFL-TBS peptide onto the LNC surface appeared to have no effect on the particle size (45.7 ± 1.4 nm), but increased significantly the PdI (0.199). With the assumption that all peptides interact with LNCs, the rate of coupled molecules was estimated around 46 peptides per LNC. In contrast to the NFL-TBS.40-63 peptide, the covalent coupling of thiolated OX26 to maleimide functions of PEG chains increased the particle size of about 18 nm (up to 63.7 ± 3.2 nm) as compared to FcdiOH–LNCs. This was in agreement with the study of [7] who reported an increase in the hydrodynamic diameter of the obtained OX26-LNCs between 12 and 30 nm [7]. The final number of bound OX26 Mabs was assessed around 1 per LNC. The drug payloads of peptide–FcdiOH–LNCs and OX26–FcdiOH–LNCs are summarised in Table 1. The decrease in concentration in FcdiOH for OX26–FcdiOH–LNCs compared to non-coated FcdiOH–LNCs resulted from dilutions during incorporation process of active ligands onto the LNC surface.

3.2. Antiproliferative effect in vitro

The MTS assay was used to determine *in vitro* cytostaticity after exposure of nanocarriers to 9L gliosarcoma cells with a cascade concentration range of FcdiOH (0.01–100 μM). The cell survival profiles of the various kinds of FcdiOH–LNCs and free FcdiOH were illustrated in Fig. 1. At low concentrations (0.01–0.1 μM), neither free FcdiOH nor FcdiOH–LNCs altered the cell growth (over 80%). Cell survival was dramatically decreased (below 20%) at the concentration

Table 1
Concentration in FcdiOH encapsulated into peptide–FcdiOH–LNCs and OX26–FcdiOH–LNCs compared to the drug payload of non-coated FcdiOH–LNCs.

Treatment	Concentration (mg/mL)
FcdiOH–LNCs	6
Peptide–FcdiOH–LNCs	6,1 (carotid) – 5,5 (CED)
OX26–FcdiOH–LNCs	3,4

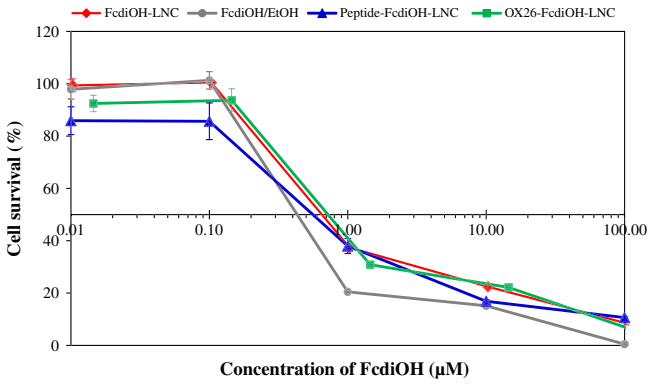

Fig. 1. Cell survival of 9L gliosarcoma cells after 72 h exposure to different treatments with free FcdiOH or with the various kinds of FcdiOH–LNCs at different concentrations of drug (0.01–100 μM). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of 10 μM, resulting in an IC_{50} of about 0.4 μM for free FcdiOH and 0.6 μM for FcdiOH–LNCs. Moreover, the incorporation of either peptides or OX26 MAbs did not compromise the cytostatic effect of FcdiOH–LNCs since their IC50 was 0.55 and 0.7 μM, respectively.

3.3. Survival studies

Peptide–FcdiOH–LNCs and OX26–FcdiOH–LNCs were administered through intra-cranial CED or carotid delivery in 9L gliosarcoma-bearing rats. Fig. 2 shows the animal survival time plotted on Kaplan–Meier curves.

We observed that CEDs of OX26–FcdiOH–LNCs and peptide–FcdiOH–LNCs significantly reduced the survival compared to the control group ($P < 0.05$). The median survival time of control rats was 25 days, whereas the medians of OX26–FcdiOH–LNC- and peptide–FcdiOH–LNC-treated rats were 22 and 11 days, respectively.

This toxicity was not observed previously after treatment with non-targeted FcdiOH–LNCs [10]. We could hypothesise that intra-tumour CED of these targeted LNCs with penetrating properties, especially for peptide–FcdiOH–LNCs, induces a large diffusion of LNCs which can affect tumour cells but also healthy cells leading to non-elucidated side effects and morbidity.

In comparison with CED, intra-carotid delivery of OX26–FcdiOH–LNCs and peptide–FcdiOH–LNCs did not induce toxicity. 9L gliosarcoma-bearing rats receiving intra-carotid injection of OX26–FcdiOH–LNCs showed a similar median survival time as control rat one. This unsuccessful treatment could be linked to the low administered dose of FcdiOH as we previously showed that antitumour efficacy of FcdiOH was dose-dependent on an orthotopic gliosarcoma model [3].Therefore, the FcdiOH concentration

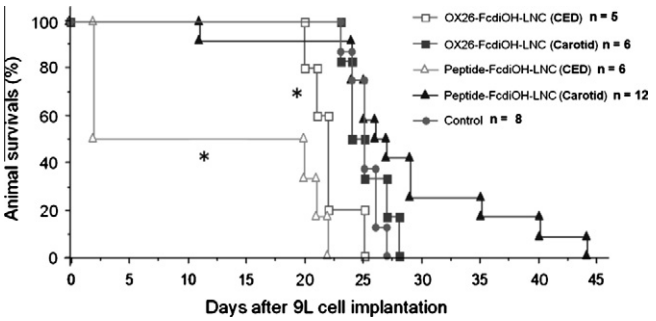

Fig. 2. Kaplan–Meier survival plots for 9L gliosarcoma-bearing rats treated with peptide–FcdiOH–LNCs and OX26–FcdiOH–LNCs by means of CED or intra-carotid injection. *Significantly different from control group ($P < 0.05$) (n = number of rats).

in the OX26–FcdiOH–LNCs might be below the pharmacologically active dose. Furthermore, the low OX26-Mabs amount coupled onto the LNCs may limit the immunonanocapsule delivery across the BBB. However, a high density of Mabs at the surface of nanoparticles is known to reduce their stealth properties [6]. In consequence, an optimal coupling rate must be achieved for a suitable brain delivery balanced with a low clearance phenomenon.

Interestingly, we observed that intra-carotid treatment with peptide–FcdiOH–LNCs increased the survival for almost half of the rats (5/12) up to 44 days. This increase in survival time had never been reached either with CED or intracarotid treatment of non-targeted LNCs [10] and represents an outstanding outcome compared to control group with the longest survival set at 27 days.

In conclusion, the treatment by intra-carotid injection with the peptide–FcdiOH–LNCs demonstrated an interesting efficacy that was strengthened by the presence of a long-term survival rat until 44 days after 9L cell implantation. This new active targeting strategy may offer a promising approach for glioma treatment, and further studies are required to ensure a safe clinical use of peptide–FcdiOH–LNCs.

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References

[1] S.D. Caruthers, S.A. Wickline, G.M. Lanza, Nanotechnological applications in medicine, Curr. Opin. Biotechnol. 18 (2007) 26–30.
[2] N.T. Huynh, C. Passirani, P. Saulnier, J.P. Benoit, Lipid nanocapsules: a new platform for nanomedicine, Int. J. Pharm. 379 (2009) 201–209.
[3] E. Allard, N.T. Huynh, A. Vessières, P. Pigeon, G. Jaouen, J.-P. Benoit, C. Passirani, Dose effect activity of ferrocifen-loaded lipid nanocapsules on a 9L-glioma model, Int. J. Pharm. 379 (2009) 317–323.
[4] W.A. Hall, Transferrin receptor on glioblastoma multiforme, J. Neurosurg. 74 (1991) 313–314.
[5] K. Ulbrich, T. Hekmatara, E. Herbert, J. Kreuter, Transferrin- and transferrin-receptor-antibody-modified nanoparticles enable drug delivery across the blood–brain barrier (BBB), Eur. J. Pharm. Biopharm. 71 (2009) 251–256.
[6] J. Huwyler, D. Wu, W.M. Pardridge, Brain drug delivery of small molecules using immunoliposomes, Proc. Nat. Acad. Sci. 93 (1996) 14164–14169.
[7] A. Béduneau, F. Hindré, A. Clavreul, J.-C. Leroux, P. Saulnier, J.-P. Benoit, Brain targeting using novel lipid nanovectors, J. Control. Release 126 (2008) 44–49.
[8] A. Bocquet, R. Berges, R. Frank, P. Robert, A.C. Peterson, J. Eyer, Neurofilaments bind tubulin and modulate its polymerization, J. Neurosci. 29 (2009) 11043–11054.
[9] G. Jaouen, S. Top, A. Vessieres, G. Leclercq, J. Quivy, L. Jin, A. Croisy, The first organometallic antioestrogens and their antiproliferative effects, C. R. Acad. Sci. Ser. IIC Chem. 3 (2000) 89–93.
[10] N.T. Huynh, C. Passirani, E. Allard-Vannier, L. Lemaire, J. Roux, E. Garcion, A. Vessieres, J.-P. Benoit, Administration-dependent efficacy of ferrociphenol lipid nanocapsules for the treatment of intracranial 9L rat gliosarcoma, Int. J. Pharm. 423 (2012) 55–62.