APPLICATION DES NANOCAPSULES LIPIDIQUES CHARGEES EN FERROCIPHENOL DANS LE TRAITEMENT DU GLIOBLASTOME

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INTRODUCTION GENERALE

1. Gliomes

Les tumeurs gliales ou gliomes concernent l'ensemble des tumeurs du cerveau issues du tissu de soutien ou *glie*. Elles représentent environ 45–50% des tumeurs primitives bénignes et malignes du cerveau [1]. Un des problèmes majeurs de la neuro-oncologie demeure le traitement des gliomes malins de par leur gravité et leur localisation. L'organisation mondiale de la santé (OMS) les repartit selon leur degré d'agressivité en différentes classes (de I à IV) [2]. Parmi eux, le glioblastome de grade IV, représente la forme la plus agressive et la plus grave de l'adulte. De plus, l'incidence des tumeurs cérébrales a augmenté remarquablement au cours des 30 dernières années et a tendance à être supérieure dans les nations développées et industrialisées [3]. En France, le registre spécifique de Gironde a enregistré une incidence annuelle de 4.96 nouveaux cas pour 100 000 habitants [4].

Dans les gliomes de bas grade, sans que l'on puisse pour autant évoquer la guérison, une exérèse complète serait associée à une survie prolongée [5]. Dans le cas des gliomes de haut grade, la chirurgie, en décomprimant le cerveau, améliore souvent l'état clinique des patients et permet de faciliter la radiothérapie mais son impact sur la survie demeure modeste et elle doit nécessairement être complétée par une irradiation et/ou une chimiothérapie [6]. Récemment, l'association de la radiothérapie externe à la chimiothérapie orale quotidienne concomitante par le témozolomide (Figure 1) a montré une réduction du risque de décès de 37% par rapport à la radiothérapie seule, avec une augmentation de la médiane de survie de 2.5 mois (passant de 12.1 mois à 14.6 mois) [7]. Les taux de survie à 2, 3, 4 et 5 ans étaient respectivement de 27, 16, 12 et 10% dans le groupe traité par témozolomide et radiothérapie contre seulement 11, 4, 3 et 2% dans le groupe traité par la radiothérapie seule [8].

Bien que les multi-thérapies permettent d'éradiquer la plupart de la masse tumorale, la récidive locale et la résistance aux thérapies antiprolifératives posent des problèmes majeurs qui entraînent fréquemment l'inefficacité des traitements. En effet, le glioblastome est une tumeur capable de s'infiltrer dans l'ensemble du cerveau et de disséminer un certain nombre de cellules malignes. Malgré des techniques neurochirurgicales de plus en performantes, les patients rechutent quasi-systématiquement avec une reprise tumorale au sein-même ou au voisinage immédiat de la tumeur initiale. En plus de ce caractère migrant, les gliomes sont constitués de cellules fortement proliférantes présentant de nombreuses altérations génétiques, ce qui les rend très résistantes aux thérapies actuelles.

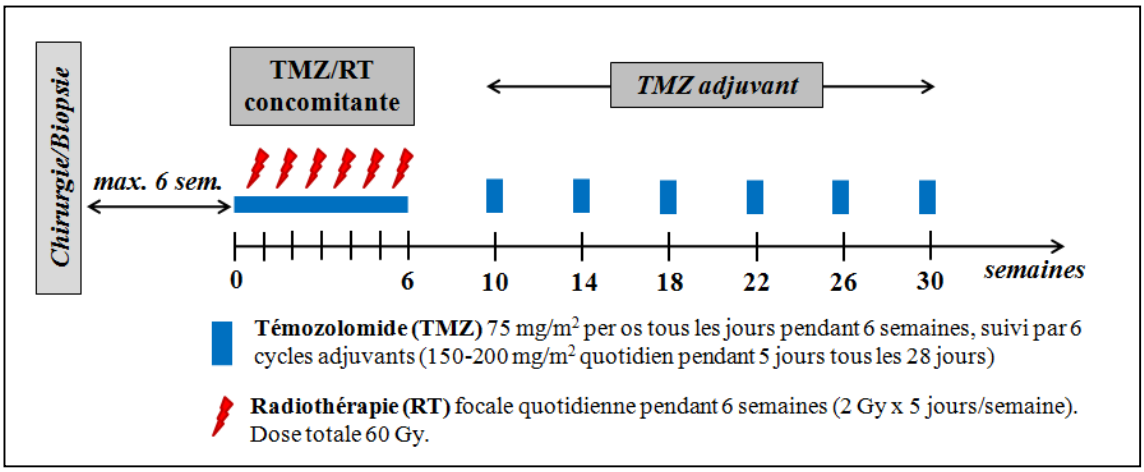

Figure 1 : Schéma de traitement du glioblastome avec le Témozolomide (reproduit de Hottinger *et al.* [9])

2. Stratégies de délivrance de la chimiothérapie

Les pré-requis principaux pour une réussite de la chimiothérapie impliquent la sensibilité des cellules cancéreuses ainsi que leur exposition suffisante vis-à-vis du principe actif administré. L'hétérogénéité de la tumeur en regard de la résistance primaire ou acquise, les paramètres pharmacocinétiques et pharmacodynamiques du principe actif ainsi que les caractéristiques du microenvironnement tumoral constituent les points clés à prendre en compte de manière spécifique dans le cas des tumeurs cérébrales [10]. Tout d'abord, la présence de la barrière hémato-encéphalique (BHE) qui isole le système nerveux central de l'organisme, représente un obstacle physiologique majeur pour une administration systémique de la chimiothérapie [11]. Par ailleurs, il faut également prendre en compte l'existence de certaines protéines de multi-résistance associées au phénomène MDR (multi-drug resistance en anglais), comme la P-glycoprotéine, surexprimée sur la BHE et favorisant l'efflux des agents thérapeutiques hors de la cellule [12]. Dans ce contexte, différentes stratégies ont été mises en place afin d'améliorer la délivrance des principes actifs dans le cerveau, notamment à l'aide de nanoparticules (Figure 2).

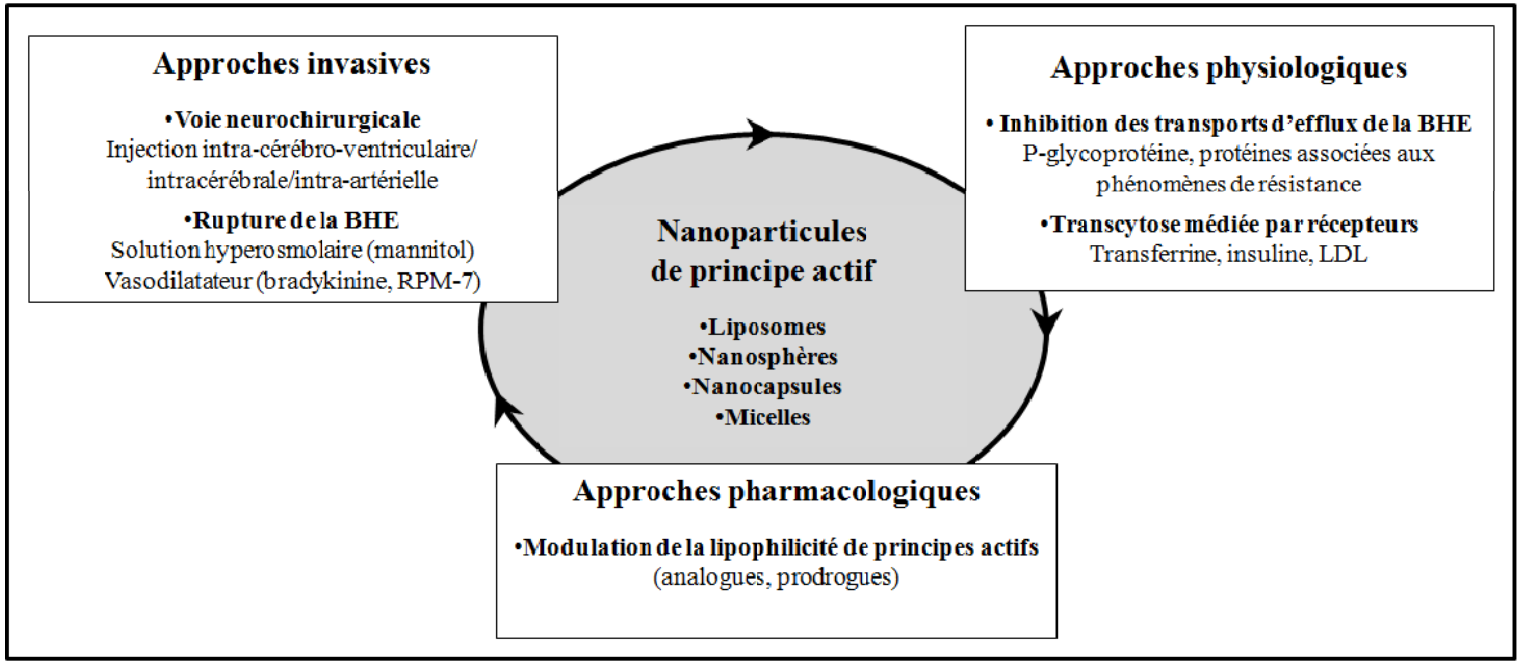

Figure 2 : Différentes approches de la chimiothérapie des cancers cérébraux à l'aide de nanoparticules

Parmi les nouvelles approches potentiellement intéressantes, les nanotechnologies offrent l'opportunité de développer des systèmes d'administration capables de diffuser parmi les cellules disséminées et de libérer le principe actif de manière prolongée, permettant ainsi une optimisation de leur potentiel thérapeutique. De plus, en variant la voie d'administration (locale, locorégionale ou systémique), les profils de biodistribution des nanoparticules pourront être modulés.

2.1.Voies d'administration

2.1.1. Administration intracérébrale

Parmi les techniques d'administration intracérébrale se distinguent principalement deux méthodes : les implants de polymères chargés en principe actif placés dans le lit tumoral après exérèse chirurgicale (administration dans une cavité) et l'injection directe de l'agent thérapeutique dans le parenchyme par stéréotaxie (en bolus ou par Convection-Enhanced Delivery (CED)).

Alors que l'implantation des polymères présente des avantages comme la libération prolongée et contrôlée du principe actif [13], cette technique est aussi caractérisée par une concentration en principe actif limitée par la taille implantée et par une faible diffusion du principe actif par gradient de concentration au sein des tissus cérébraux.

La stéréotaxie est une technique de neurochirurgie permettant, associée à l'IRM (imagerie par résonance magnétique), de localiser et atteindre précisément les différentes zones du cerveau. Elle nécessite l'utilisation d'un système de contention qui est aussi un équipement de repérage, appelé cadre stéréotaxique, fixé sur la tête du patient. Un atlas stéréotaxique de référence permet de déterminer les coordonnées précises dans les 3 plans orthogonaux des différentes structures du cerveau. L'injection d'un bolus par stéréotaxie consiste en l'administration rapide d'un fluide, entrainant la diffusion passive des principes actifs à partir du site d'injection. Par conséquent, les limites principales sont une faible distribution des principes actifs et le risque de reflux le long de l'aiguille lors de l'injection. La CED est décrite pour la première fois par Bobo et al [14]. Cette technique consiste en l'injection stéréotaxique continue d'un fluide contenant les agents thérapeutiques, via un cathéter connecté à un pousse-seringue, sous pression positive. Dans ce cas, l'agent thérapeutique est

délivré principalement par convection, phénomène physique qui s'ajoute à la simple diffusion et qui est indépendant de la masse moléculaire. Ainsi, la CED permet de délivrer des principes actifs dans le cerveau à de fortes concentrations, et au travers de larges volumes de distribution tissulaire, tout en réduisant les risques de toxicité systémique [15].

2.1.2. Administration intra-carotidienne

Une approche alternative efficace proche de la délivrance directe du principe actif dans le cerveau est l'administration intra-carotidienne [16]. Le principe actif est administré directement dans l'artère et orienté vers la vasculature cérébrale avant de rentrer dans les tissus, en évitant l'effet de premier passage périphérique. L'injection intra-carotidienne peut être considérée comme une délivrance locorégionale qui permet d'augmenter la concentration des principes actifs dans la vascularisation tumorale [17].

Ces dernières années, de rapides avancées dans le domaine de la neurochirurgie endovasculaire a permis d'améliorer les techniques d'injection intra-carotidienne en diminuant les risques de complications neurologiques ainsi qu'en améliorant significativement le dépôt des agents thérapeutiques dans les tissus cibles [18]. L'IRM de résolution temporelle permettant de guider la mise en place d'un cathéter, décrite comme "imagerie par résonance magnétique interventionnelle", progresse très rapidement. De nouvelles interventions intra-artérielles assistées par IRM se développent actuellement [19]. De vastes études sur l'animal ainsi que sur l'homme ont montré les bénéfices de l'administration intra-carotidienne dans le traitement du cancer cérébral [20-25].

2.1.3. Administration intraveineuse

L'injection intraveineuse constitue une voie d'administration conventionnelle et appropriée à la chimiothérapie. En effet, c'est une méthode non-invasive, de manipulation simple, permettant de diminuer les traumatismes ainsi que les frais neurochirurgicaux. De plus, sous certaines conditions, l'agent thérapeutique va se disséminer dans le corps grâce à la circulation sanguine et pouvoir atteindre certains tissus tels que les métastases. Dans le cas des glioblastomes, grâce à la rupture locale spécifique de la BHE au niveau de la masse

tumorale, la délivrance d'agents anticancéreux via une injection intraveineuse peut être atteinte mais, l'efficacité de cette méthode est variable et controversée [26-29].

3. Nanocapsules lipidiques (LNC)

Parmi les systèmes nanoparticulaires de principes actifs, notre équipe s'intéresse tout particulièrement à la conception de vecteurs colloïdaux biomimétiques, les nanocapsules lipidiques appelées LNC. La conception de ces particules, parfaitement stables sur le plan physique, et dont la taille peut s'échelonner de 20 à 100nm, repose sur un phénomène d'inversion de phase d'une micro-émulsion [30]. Les LNC sont constituées d'un cœur liquide de triglycérides à chaînes moyennes (Labrafac® WR 1349), entouré par des phospholipides (Lipoïd® S75-3) et des polymères hydrophiles de type poly(éthylène glycol) (PEG) (Solutol® HS15 – PEG 660). L'ensemble est introduit dans une phase externe aqueuse d'eau MiliQ® contenant du sel de chlorure de sodium NaCl. De nombreux composés lipophiles ont pu être encapsulés dans le cœur lipidique des LNC permettant ainsi de constituer une réelle plateforme de nanomédicaments ([31] : revue bibliographique n°1).

Ces LNC peuvent être recouvertes de longues chaînes de PEG (2000 kDa) à leur surface par une technique simple de post-insertion. Ce recouvrement permet aux LNC pégylées d'échapper à la capture prématurée par les macrophages du sang après une injection systémique et donc d'acquérir un temps de demi-vie plasmatique prolongé. Ce phénomène favorise l'accumulation passive dans les tumeurs grâce à l'effet de perméabilité et de rétention tissulaire, encore appelé effet EPR (enhanced permeability and retention) [32-33]. Récemment, les LNC recouvertes par le 1,2-distéaroyl-sn-glycéro-3-phosphoéthanolamine-N-[méthoxy(polyéthylène glycol) 2000] (DSPE-mPEG2000) ont montré une prolongation de la durée de résidence plasmatique et une accumulation importante dans la tumeur sous-cutanée de gliome humain U87MG, après une injection intraveineuse chez la souris [34]. De plus, les PEG post-insérés à la surface des LNC possèdent des fonctions chimiques terminales permettant le greffage de structures de reconnaissance. Cette réactivité peut être mise à profit pour greffer des ligands spécifiques, comme par exemple des anticorps, des haptènes, des peptides, des sucres ou tout autre type de ligand capable de favoriser le passage des

nanovecteurs à travers certaines barrières physiologiques, telle que la BHE. Ces ligands de ciblage peuvent également favoriser l'internalisation des vecteurs au sein des cellules tumorales [35]. Par exemple, le greffage des anticorps monoclonaux OX26 à la surface des LNC pégylées (nommées alors immunonanocapsules) a montré une augmentation de la concentration des LNC dans le cerveau d'animaux sains [36].

4. Ferrociphénol (FcdiOH)

Depuis le succès du cisplatine utilisé dans le traitement du cancer depuis une quarantaine d'années, les recherches sur les dérivés organométalliques n'ont cessé de progresser. La synthèse de nouvelles molécules ainsi que la création de systèmes innovants permettant leur délivrance spécifique au niveau des tissus d'intérêt est en pleine expansion [37-40].

En collaboration avec le Pr. Gérard Jaouen et le Dr. Anne Vessières de l'unité UMR 7223 de Paris, notre équipe de recherche s'intéresse aux dérivés du tamoxifène dont l'un des groupements phényle est substitué par un groupement ferrocène [$Fe(C_5H_5)_2$]. Tandis que le tamoxifène est bien connu pour son activité hormonodépendante, ces nouveaux composés dénommés « ferrocifènes » présentent un effet antiprolifératif considérable sur des lignées de cultures hormono-dépendantes (MCF-7) mais également hormono-indépendantes (MDA-MB231) [41]. L'efficacité supposée du FcdiOH est basée sur deux oxydations intramoléculaires successives, la création de radicaux libres et la formation de quinone méthide (Figure 3). Cette molécule, à son tour, serait capable d'interagir avec des macromolécules telles que le glutathion, l'ADN ou certaines protéines pour aboutir à une mort cellulaire par vieillissement accéléré (sénescence) [42].

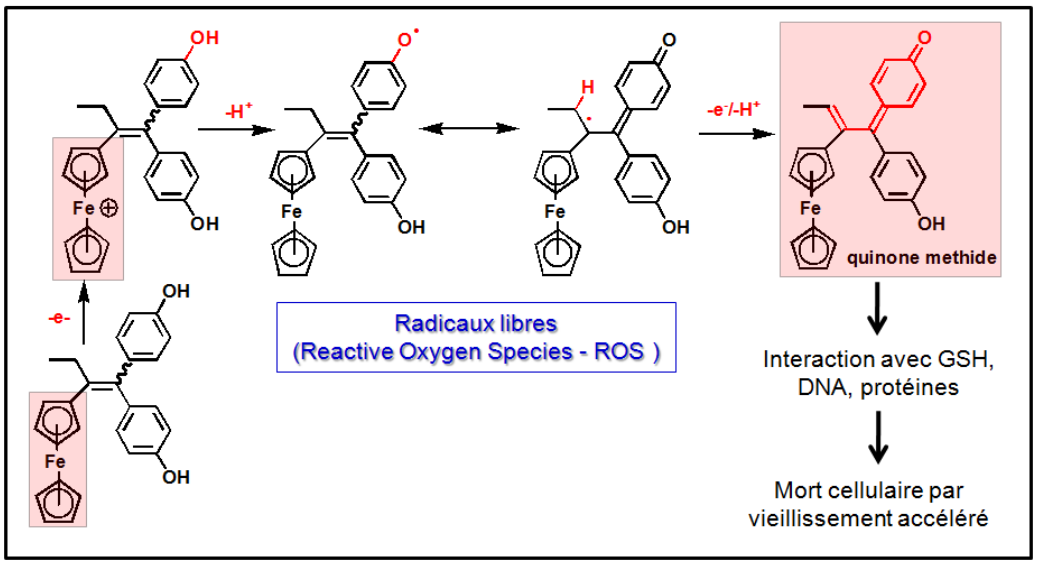

Figure 3 : Mécanisme d'action proposé des ferrocifènes.

Parmi ces molécules, le composé de 2-ferrocényl-1,1-bis(4-hydroxyphényl)-but-1-ène , ou ferrociphénol (FcdiOH) (Figure 4) s'est montré très actif *in vitro* sur les cellules de gliome 9L avec un IC50 de 0,5 µM. Par ailleurs, sa toxicité est largement inférieure sur les astrocytes (IC50 = 50 µM) [43] suggérant que le FcdiOH est toxique sur les cellules tumorales présentant une forte division cellulaire mais inoffensif sur les cellules saines. Enfin, cette molécule présentant de forte propriétés hydrophobes, son encapsulation au sein des LNC va être nécessaire afin d'améliorer sa biodisponibilité *in vivo* [44].

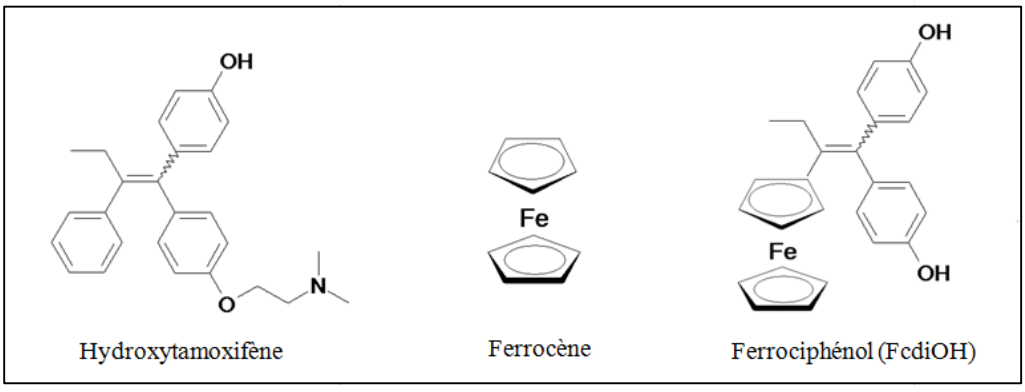

Figure 4 : Structure chimique de l'hydroxytamoxifène, du ferrocène et du ferrociphénol (FcdiOH).

5. Travaux antérieurs concernant les LNC-FcdiOH

Les études précédentes réalisées par Emilie Allard (Thèse de doctorat, Université d'Angers, 2008) ont montré une bonne capacité d'encapsulation du FcdiOH au sein des LNC à une concentration élevée allant jusqu'à 2% w/w en poids sec, correspondant à 6,5 mg de FcdiOH par gramme de suspension. Les rendements d'encapsulation de cette molécule hydrophobe sont toujours supérieurs à 98% [43]. L'activité du FcdiOH est conservée après son encapsulation sur des cellules 9L et l'internalisation des LNC a été mise en évidence par fluorescence. Ces vecteurs, administrés en intra-tumoral chez le rat dans un modèle de gliome sous-cutané, ont permis de diminuer significativement à la fois la masse et le volume de la tumeur. Ceci a mis en évidence pour la première fois une efficacité *in vivo* de ce genre de composé bio-organométallique. Par ailleurs, dans un modèle orthotopique de gliome intracérébral, l'administration locale de LNC-FcdiOH (0.36 mg/rat) par CED, en association avec les photons X délivrés par radiothérapie externe, a montré un effet synergique mettant en évidence les propriétés radio-sensibilisantes des ferrocifènes [45]. En effet, le groupe de rat traité par une CED de LNC-FcdiOH suivi d'une radiothérapie externe de 18Gy (3x6Gy) a donné lieu à une médiane de survie augmentée de 60 % par rapport au groupe contrôle (40 jours par rapport à 25) avec la présence de 10% de longs survivants (> 100 jours). En parallèle, afin d'optimiser la convection, du sucrose a été ajouté en phase externe pour augmenter la viscosité des suspensions colloïdales, réduisant les risques de reflux et augmentant ainsi les volumes de distribution [46-47]. Malheureusement, la chimiothérapie seule avec les LNC-FcdiOH préparées dans ces conditions n'a montré aucune efficacité, voire de la toxicité, puisque la médiane de survie des rats est devenue inférieure à celle du groupe témoin (23 jours au lieu de 25).

6. Objectif de thèse

Ce travail de thèse s'inscrit dans une stratégie d'optimisation de la chimiothérapie seule à l'aide des LNC chargées en FcdiOH, molécule organométallique innovante, dans le traitement des gliomes. Dans cet objectif, plusieurs axes ont été envisagés afin de développer un traitement anticancéreux optimal appliqué au glioblastome, en étudiant à la fois plusieurs

voies d'administration et différentes propriétés de ciblage apportées par les nanocapsules lipidiques.

La première partie s'intéresse à l'administration locale et locorégionale des LNC et se divise en 3 chapitres. Le premier est une revue bibliographique décrivant la conception des LNC, leur intérêt et les stratégies d'application en chimiothérapie avec différents types de principes actifs anticancéreux. Le deuxième étudie l'effet de la dose administrée de FcdiOH dans un modèle orthotopique de gliosarcome 9L chez le rat. Enfin, le troisième chapitre concerne l'évaluation des effets secondaires et de l'efficacité des LNC-FcdiOH administrées par CED ou par voie intra-carotidienne dans le traitement de la tumeur intracérébrale.

La deuxième partie s'organise également en 3 chapitres consacrés à l'optimisation des propriétés thérapeutiques des LNC par ciblage passif ou actif. Le premier est une revue bibliographique discutant des avantages et inconvénients du ciblage actif par rapport au ciblage passif. Le deuxième consiste à évaluer les propriétés furtives des LNC-FcdiOH pégylées ou non et leurs effets antitumoraux, après injection intraveineuse dans un modèle ectopique ainsi qu'un modèle orthotopique de gliosarcome. Le troisième et dernier chapitre de ce travail constitue une étude préliminaire vers un ciblage actif des cellules tumorales à l'aide du peptide internalisant NFL-TBS, incorporé à la surface des LNC-FcdiOH.

A la fin, une discussion générale permet de synthétiser l'ensemble des résultats et d'apporter des informations complémentaires par rapport aux publications présentées dans les deux premières parties. Cette discussion tentera de faire ressortir les points forts de nos nanovecteurs afin d'ouvrir de nouvelles perspectives dans le traitement du glioblastome.

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PARTIE 1

PARTIE 1

Traitement local et locorégional des gliomes à l'aide des LNC-FcdiOH

En raison de la fragilité de l'environnement intracérébral, la biocompatibilité des vecteurs utilisés pour la chimiothérapie est un pré-requis indispensable dans le traitement des gliomes. Notre laboratoire a développé et breveté un système nanométrique monodisperse, stable et reproductible : les nanocapsules lipidiques (LNC). Ces nanovecteurs sont constitués d'excipients biocompatibles et/ou biodégradables, tous approuvés par la Food and Drug Administration (FDA). Par ailleurs, la préparation des LNC passe par un procédé n'ayant recours à aucun solvant organique et sans consommation importante d'énergie.

Cette partie décrit, d'une part, la conception et la formulation des LNC ainsi que leur application en tant que vecteurs destinés à l'administration de molécules lipophiles (revue bibliographique n°1). D'autre part, l'administration locale et locorégionale des LNC-FcdiOH dans le traitement du glioblastome est évaluée, après optimisation des doses (publication de résultats n°1) et des conditions d'injection (publication de résultats n°2).

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Review

Lipid nanocapsules: A new platform for nanomedicine

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ABSTRACT

Nanomedicine, an emerging new field created by the fusion of nanotechnology and medicine, is one of the most promising pathways for the development of effective targeted therapies with oncology being the earlier and the most notable beneficiary to date. Indeed, drug-loaded nanoparticles provide an ideal solution to overcome the low selectivity of the anticancer drugs towards the cancer cells in regards to normal cells and the induced severe side-effects, thanks to their passive and/or active targeting to cancer tissues. Liposome-based systems encapsulating drugs are already used in some cancer therapies (e.g. Myocet, Daunoxome, Doxil). But liposomes have some important drawbacks: they have a low capacity to encapsulate lipophilic drugs (even though it exists), they are manufactured through processes involving organic solvents, and they are leaky, unstable in biological fluids and more generally in aqueous solutions for being commercialized as such. We have developed new nano-cargos, the lipid nanocapsules, with sizes below the endothelium fenestration (ϕ < 100 nm), that solve these disadvantages. They are prepared according to a solvent-free process and they are stable for at least one year in suspension ready for injection, which should reduce considerably the cost and convenience for treatment. Moreover, these new nano-cargos have the ability to encapsulate efficiently lipophilic drugs, offering a pharmaceutical solution for their intravenous administration.

The lipid nanocapsules (LNCs) have been prepared according to an original method based on a phase-inversion temperature process recently developed and patented. Their structure is a hybrid between polymeric nanocapsules and liposomes because of their oily core which is surrounded by a tensioactive rigid membrane. They have a lipoprotein-like structure. Their size can be adjusted below 100 nm with a narrow distribution. Importantly, these properties confer great stability to the structure (physical stability > 18 months). Blank or drug-loaded LNCs can be prepared, with or without PEG (polyethyleneglycol)ylation that is a key parameter that affects the vascular residence time of the nano-cargos. Other hydrophilic tails can also be grafted. Different anticancer drugs (paclitaxel, docetaxel, etoposide, hydroxytamoxifen, doxorubicin, etc.) have been encapsulated. They all are released according to a sustained pattern. Preclinical studies on cell cultures and animal models of tumors have been performed, showing promising results.

Contents

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1. Introduction

Research and development (R&D) of new chemical or biological entities applied in therapeutics is a multimodal field requiring continuous commitment to conduct worldwide, scientific research. Globally, pharmaceutical R&D expenditure has increased continually year by year. However, according to data from the FDA (Food and Drug Administration), the number of new molecular entities (NMEs) approved by the FDA is decreasing. 53 NMEs received FDA approval in 1996, 27 in 2000, and only 22 in 2006. At the same time, major patents for blockbuster drugs will arrive at the end of their validity over the next few years, and the pharmaceutical industry is currently exploring a drug strategy to reposition existing active ingredients. One striking example is the case of sildenafil (VIAGRA®), the first selective-type 5-phosphodiesterase inhibitor. It was initially studied for use in angina pectoris. Interestingly, the results of the first clinical trial suggested that the drug had little effect on angina, but that it could induce marked penile erections (Osterloh and Riley, 2002; Ghofrani et al., 2006) so that it has now become an effective 'on demand' treatment of erectile dysfunction in men. Thus, this "repositioning" strategy leading to the discovery of new indications, new targets and also new drug delivery systems constitutes the foundation of nanomedicine which has attracted considerable interest in the recent years as an emerging new field created by the fusion of nanotechnology and medicine. Indeed, it can provide medical and pharmaceutical benefits, especially in oncology, because it enables the control of drug characteristics such as solubility, vascular circulation time, and specific site-targeted delivery (Caruthers et al., 2007). Thanks to their passive and/or active targeting of cancer tissue, drug-loaded nanoparticles provide an ideal solution, leading to selective cytotoxicity in the targeted tumor cells while also preventing harm to healthy cells. In this way, many anticancer agents have been incorporated into nanoparticles and have so far gained some success in research in the field of therapeutic application (Working and Dayan, 1996; Steiniger et al., 2004; Haley and Frenkel, 2008).

Taking this "repositioning" strategy in consideration, a number of colloidal drug delivery systems with a size below 100 nm have been designed to encapsulate the drug in carriers including micelles, different types of liposomes, nanoparticles made from polymers or lipids, polymer–drug conjugates, dendrimers, . . . (Letchford and Burt, 2007). Among these carriers, our research group has recently developed and patented biomimetic carriers that mimic lipoproteins: lipid nanocapsules (LNCs) (Table 1) (Heurtault et al., 2000). Their size ranges from 20 to 100 nm, and they are characterized by a hybrid structure between polymer nanocapsules and liposomes. As compared to liposomes which manufacture through processes involving organic solvent and are leaky, unstable in biological fluids, LNCs are prepared by a solvent-free, soft-energy procedure and present a great stability (with physical stability up to 18 months). They have generally an oily core, corresponding to medium-chain triglycerides surrounded by a membrane made from a mixture of lecithin and a pegylated surfactant. Their formulation is based on the phase-inversion temperature phenomenon of an emulsion leading to lipid nanocapsule formation with good mono-dispersion (Heurtault et al., 2002a).

In this review, we will focus on LNCs with a description of their preparation process and their physical characteristics. We will also discuss the various strategies used in drug delivery for cancer treatment using LNCs as potential targeting carriers.

2. LNC formulation

2.1. Preparation

LNC formulation is based on at least three principal components: an oily phase, an aqueous phase and a nonionic surfactant. The oily phase is essentially constituted of triglycerides of capric and caprylic acids known under the commercial name of Labrafac® WR 1349. The hydrophilic surfactant, Solutol® HS 15, is derived from polyethyleneglycol (PEG) and is a mixture of free PEG 660 and PEG 660 hydroxystearate. The aqueous phase consists of MiliQ® water plus sodium chloride salt, NaCl. Furthermore, another surfactant, Lipoid®, composed of 69% phosphatidylcholine soya bean lecithin, is used in small proportions to significantly increase LNC stability (Minkov et al., 2005; Vonarbourg et al., 2005), which is especially necessary in the case of 50–100 nm LNC formulations. All components are approved by the FDA for oral, topical and parenteral administration. Each component has different influences on LNC formulation and stability which are cited in Table 2.

According to the patent No. WO02688000 (Heurtault et al., 2000), the preparation process of this type of lipid nanocapsule involves two steps. Step I consists in mixing all the components (whose proportions vary according to the study) under magnetic stirring and heating from room temperature up to T2 temperature, above the phase-inversion temperature (PIT), to obtain a W/O emulsion. This is followed by a cooling process to the T1 temperature, below the PIT, leading to the formation of an O/W emulsion. Several temperature cycles crossing the phase-inversion zone (PIZ) between T2 and T1 are then carried out. The temperature before

Table 1
Patents related to LNC formulation.

Patent	Authors	Number
Lipidic nanocapsules: preparation process and use as drug delivery systems	Saulnier, P., Heurtault, B., Benoit, J.P., Proust, J.E., Pech, B., Richard, J.	WO02688000
Nanocapsules with liquid lipidic core loaded with water-soluble or water-dispersible ingredient(s)	Anton, N., Saulnier, P., Benoit, J.P.	WO2009001019
Method for preparing lipid nanoparticles	Benoit, J.P., Anton, N., Saulnier, P.	WO2009004214
Aqueous core lipidic nanocapsule encapsulation and release of fragile hydrophilic et/ou lipophilic drugs	Anton, N., Saulnier, P., Benoit, J.P.	PCT/EP2008/062435
Nanocapsules of lipophilic complexes of nucleic acids	Saulnier, P., Benoit, J.P., Passirani, C., Vonarbourg, A., Lambert, O., Pitard, B.	WO2008096321

Table 2
Factors influencing the formulation and the stability of LNC prepared by phase-inversion temperature (PIT) method.

Factors	Effects	Reference
Nonionic surfactant amount (Solutol®)	Major influence on LNC formation and stability	Heurtault et al. (2002b, 2003c), Anton et al. (2007a)
Temperature cycles	Favoring LNC formation and improving the quality of LNC dispersion	Anton et al. (2007a, 2008)
Oil proportions (Labrafac®)	Increase of LNC size	Heurtault et al. (2003c)
NaCl	Decrease of PIT	Heurtault et al. (2002a), Anton et al. (2007b)
Lipophile surfactant (Lipoid®)	Stabilizing the LNC rigid shell and favoring the freeze-drying process	Dulieu and Bazile (2005), Vonarbourg et al. (2005)

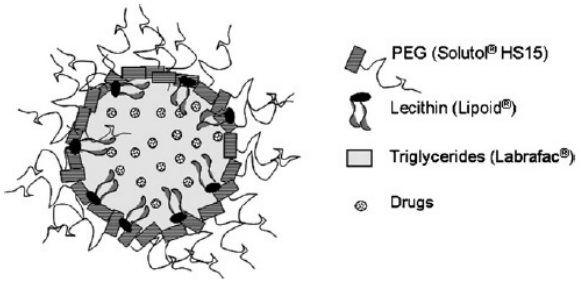

Fig. 1. Schematic representation of LNC prepared by the phase-inversion temperature method.

dilution is determined at the beginning of the inversion process and is defined by a temperature range that is set at 1–3 °C from the beginning of the O/W emulsion. Step II is an irreversible shock, induced by sudden dilution with cold water added to the mixture which has been maintained at the previously defined temperature. This is done to break the microemulsion system obtained in the PIZ, and leads to the formation of stable nanocapsules. Afterwards, slow magnetic stirring is applied to the suspension for 5 min. Different composition proportions are prepared in order to explore the phase diagram (Heurtault et al., 2003c). Three temperature cycles of heating and cooling at the rate of 4 °C/min are usually applied between 85 and 60 °C (Heurtault et al., 2002a; Saulnier et al., 2008).

The above-mentioned process leads to the formation of lipid nanocapsules constituted of an oily core, corresponding to free Labrafac®. The tensioactive, cohesive membrane is made up of the mixture of Lipoid® anchored in the oily phase, and Solutol® oriented towards the water phase (Fig. 1) (Heurtault et al., 2003a).

2.2. PIT process—technological principles

The PIT method was first introduced by Shinoda and Saito (1969) and is now widely used in industry. PIT is defined as the "temperature or temperature range at which the hydrophilic and lipophilic properties of a nonionic surfactant just balance" (Friberg et al., 1976). This method is essentially based on the changes in solubility of polyoxyethylene-type, nonionic surfactants according to the temperature. These types of surfactant become lipophilic with increasing temperature as a consequence of the dehydration of polyoxyethylene chains due to the breakdown of hydrogen bonds with water molecules. At low temperatures, the surfactant monolayer has a large, positive, spontaneous curvature forming O/W emulsions, characterized by high conductivity values (35 mS/cm). By increasing the temperature, the spontaneous curvature becomes negative, leading to W/O emulsion formation as a rapid decrease in conductivity of nearly 0 mS/cm at 84 °C is observed. This suggests a phase-inversion process from an O/W to a W/O emulsion which takes place in the PIZ. This zone demonstrates a continuous variation of conductivity between W/O and O/W emulsions attributed to bicontinuous microemulsion structures in which the spontaneous curvature becomes close to zero (Morales et al., 2003; Solans et al., 2005).

The PIZ temperatures are strongly affected by salinity levels. Generally, the more sodium chloride added, the higher the conductivity (Miller et al., 2001). This directly influences the temperature of the first rise in conductivity (T_{rise}) during the cooling of the W/O emulsion, by modifying the solubility of the polyethoxylated surfactant (Anton et al., 2007b). T_{rise} decreases as the NaCl concentration increases, but the PIZ remains virtually unchanged. This provides the possibility to modify the temperature range, with salinity being of prime importance for the encapsulation of thermolabile drugs (Heurtault et al., 2002a). Furthermore, drug degradation is expected to be limited because of the short heating period. Indeed, an *in vitro* study on the effect of etoposide-loaded LNCs (Lamprecht and Benoit, 2006), a drug which possesses poor aqueous solubility and chemical instability (Shah et al., 1989), on glioma cell growth demonstrated that this technique preserved not only the integrity but also the activity of the drug.

2.3. Nanoemulsions and nanocapsules

Nanoemulsions are nanometric-scale emulsions, typically displaying droplet diameters in the range of 20–200 nm (Solans et al., 2005). In contrast to microemulsions that are thermodynamically stable systems that form spontaneously, nanoemulsions are only stable kinetically. Two fundamental processes may be applied for the preparation of nanoemulsions, either by high-energy emulsification methods (e.g. high pressure homogenizers (Floury et al., 2003) or ultrasound generators (Abismail et al., 1999)) or by low-energy methods (e.g. spontaneous emulsification (Bouchemal et al., 2004) or the phase-inversion temperature (PIT) process (Anton et al., 2007a)).

Both nanoemulsions and nanocapsules can be prepared by the PIT technique, generating some objects in the nanoscale range. To discriminate one from another, it is useful to review the ternary diagram consisting of different proportions of aqueous phase, oil phase and nonionic surfactant, and also the prismatic diagram obtained by adding the temperature parameter (Fig. 2a). The characterization of an entire prism may become complicated. However, this can be simplified by keeping constant either the concentration of nonionic surfactant, corresponding to χ in the diagram, or the water/oil ratio (WOR), corresponding to γ in the diagram. The latter represents the Fish-cut diagram which allows the Winsor system to be defined under equilibrium conditions (Winsor, 1954). As far as the temperature (T) axis is concerned, at low T levels, over the Winsor I region, O/W emulsions are formed; at higher T levels, the Winsor II region corresponds to the formation of W/O emulsions; at the intermediate T or PIT, the system is balanced; in this situation, the Winsor III region (consisting of water, microemulsion, and oil phases in equilibrium) or the Winsor IV region (single phase microemulsion) occurs (Fig. 2b) (Morales et al., 2003). Depending on the amount of surfactant, phase equilibrium may be found either in the Winsor III or in the Winsor IV region, generating different final formulations. With small amounts of nonionic surfactant, the three-phase region is observed. Near the PIT, this system has the minimum degree of interfacial tension. A sudden dilution in cold water of this system produces nanoemulsions. Conversely, with larger quantities of surfactant (>10 wt.%), when combined with a temperature

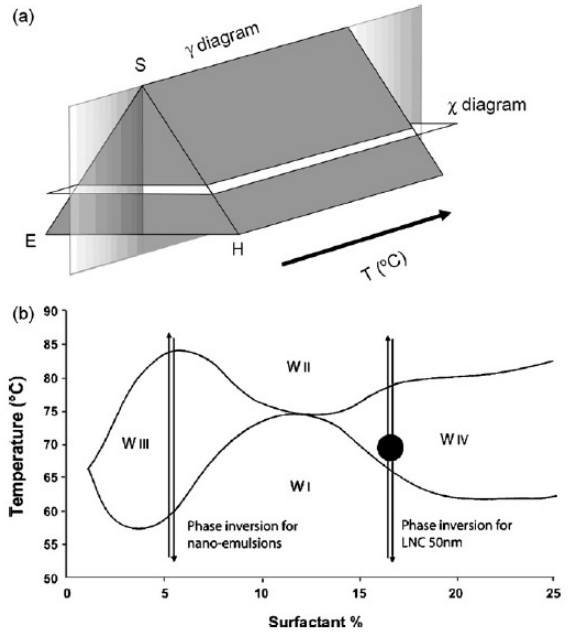

Fig. 2. Prismatic diagram (a) representing the correlation among proportions of aqueous phase, oil phase, nonionic surfactant amount and temperatures. At a constant water/oil ratio, the γ diagram or Fish-cut diagram (b) is obtained and allows determining the Winsor system under equilibrium conditions in the function of temperature and of surfactant amount.

cycling process, the rapid cooling of the Winsor IV system leads to the formation of lipid nanocapsules (Anton et al., 2008).

Since the temperature of this final formulation is below the nonionic surfactant melting point (about 30 °C), shell crystallization can occur; this prevents from the coalescence of the droplets and leads to the formulation of stable LNC suspensions at room temperature (Dulieu and Bazile, 2005). Thanks to this rigid shell, such suspensions allow freeze-drying to take place by means of adding a cryoprotectant, such as mannitol, glucose or trehalose. Among the tested cryoprotectants, trehalose provides the best polydispersity index of the sample after freeze-drying and resuspension (Heurtault et al., 2002a; Dulieu and Bazile, 2005). On the contrary, nanoemulsions are only stable kinetically, and one of the main instability problems of nanoemulsions involves the increase of the Ostwald ripening rate under storage. Ostwald ripening results from the difference in interfacial tension between small and large droplets, causing the diffusion of oil molecules from small to larger droplets (Tadros et al., 2004).

2.4. Physical characteristics

LNC particle size and dispersity are strongly dependent on the proportions of the constituents. A ternary diagram was established to optimize the constituent proportions before the cooling dilution takes place (Heurtault et al., 2002a, 2003c). By fixing the proportions of NaCl in water at 1.75% and of Lipoid® at 1.5%, a feasibility domain was determined as a parallelogram whose relative proportions were comprised approximately from 10 to 40% of hydrophilic surfactant, 35 to 80% of water, and 10 to 25% of oil (Fig. 3). Once nanoparticles are formed, their average volume size ranges from 20 to 100 nm, and exhibit a very narrow range of dispersity (PdI < 0.3).

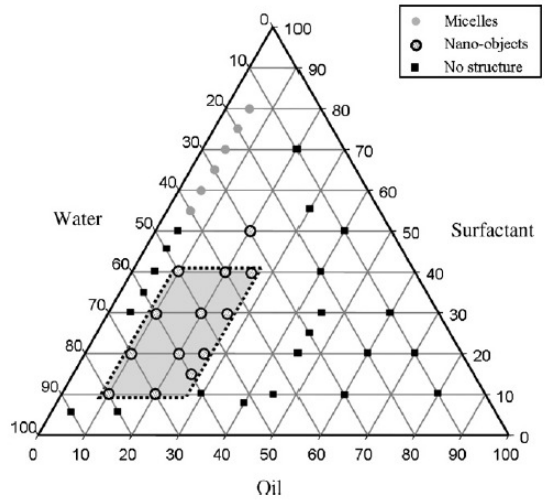

Fig. 3. The feasibility zone for the formation of LNC corresponding 10–40% of hydrophilic surfactant, 35–80% of water and 10–25% of oil by fixing the amount of NaCl at 1.75% and of lipophilic surfactant (Lipoid®) at 1.5% (inspired from (Heurtault et al., 2003c)).

In the region of feasibility, the percentage of hydrophilic surfactant (Solutol®) has a major effect on the average LNC diameters because, if it is increased, it leads to a considerable decrease of average particle diameter. This phenomenon results from its properties at the triglyceride/water interface (Heurtault et al., 2002b). Conversely, an increase of oil proportion leads to an increase of particle size whereas the proportion of water has no effect on particle diameter.

Furthermore, the temperature cycling process crossing the PIZ plays a relatively important role on LNC formulation. An increasing number of cycles favors LNC formation and improves the quality of LNC size and dispersion. Concretely, the less the amount of surfactant added, the higher the number of temperature cycles required to stabilize nanometric dispersion. For the larger amounts of surfactant, several cycles do not really appear to be necessary (Anton et al., 2007a). Within the feasibility zone, an increase of the number of temperature cycles to over 3 does not really seem to be beneficial to LNC size and polydispersity index reduction.

The zeta potential represents the electric potential at the LNC shear plane. This is an important and useful indicator to predict and control LNC stability (Heurtault et al., 2003b). The measurement of zeta potential is performed by means of a laser Doppler method. In general, LNCs have a negative surface charge due to the negative contribution of phospholipids molecules (Manconi et al., 2003) and the presence of PEG dipoles in their shell (Vonarbourg et al., 2005).

3. LNC development strategies

3.1. Drug-loading into LNCs and their release profiles

The existence of such nanoparticulate formulations provides an opportunity to encapsulate various kinds of molecules designed for anti-infection or anticancer action using various strategies for drug delivery to the tumor. Moreover, these nano-cargos present an efficient drug-loading mechanism with the encapsulation rates over 90% (Table 3). This encapsulation yield is much higher than that of liposomes (about 50%) (Wehrlé, 2007).

Firstly, amiodarone, an antiarrhythmic drug used in heart disease, was studied as a model drug encapsulated into LNCs (Lamprecht et al., 2002). Amiodarone was attracting attention

Table 3
Various strategies for drug delivery to the sites of action using LNC.

Strategies	Examples	Encapsulated drugs	Encapsulation rates	Study designs	Results	Reference
P-gp inhibition	LNC coated with PEG-type nonionic surfactants such as Solutol®	Etoposide	89.9 ± 2.3%	*In vitro* on C6, F98, 9L glioma cell lines	Increase cytotoxicity on glioma cells due to high intracellular drug accumulation	Lamprecht and Benoit (2006)
		Paclitaxel	93.0 ± 3.1%	*In vitro* on 9L and F98 glioma cell lines *In vivo* on s.c. F98 tumor model, single i.t. treatment at Day 5	Significant reduction in cell survival Significant reduction in tumor mass and tumor volume evolution	Garcion et al. (2006)
Passive targeting	Post-insertion of longer PEG chains: DSPE-PEG 1500; DSPE-PEG 2000; DSPE-PEG-5000	Drug-free		Biodistribution after an i.v. injection into healthy rats	Half-life time over 5h *vs* under 21 min for conventional LNC	Hoarau et al. (2004), Ballot et al. (2006), Beduneau et al. (2006)
	Post-insertion of DSPE-PEG 2000	Docetaxel	>98%	C26 colon adenocarcinoma s.c. tumor, i.v. injection of treatments in mice	Significant and substantial accumulation in the tumor *vs* conventional LNC and control docetaxel formulation (Taxotere®)	Khalid et al. (2006)
Active targeting	Attachment of OX26 Mab or Fab′ fragments at the LNC surface directed against TfR	Drug-free		*In vitro* cell binding on Y3.AG.1.2.3. cells and rat BCECs	Effective binding of immuno-nanocapsules on the cells *via* TfR	Beduneau et al. (2007a,b)
				Biodistribution after an i.v. injection into healthy rats	Significant accumulation in the brain 24h after administration *vs* non-targeted LNC	
Local administration (CED)	CED technique for delivery of LNC into the brain	^{188}Re-SSS; Fc-diOH	>98%	9L rat brain tumor intracranial xenograft model, CED treatment	Significant improvement in median survival time	Allard et al. (2008a)
Oral administration	LNC formulation to inhibit P-gp on the gastrointestinal tract	Paclitaxel	99.9 ± 1%	Oral administration by gastric intubation into healthy rats	Augmentation of mean plasmatic concentration of paclitaxel	Peltier et al. (2006)

P-gp: P-glycoprotein; LNC: lipid nanocapsules; PEG: polyethyleneglycol; s.c.: subcutaneous; i.t.: intratumoral; i.v.: intravenous; Mab: monoclonal antibodies; TfR: transferrin receptor; BCECs: brain cerebral endothelial cells; CED: convection-enhanced delivery; ^{188}Re-SSS: $^{188}Re(S_3CPh)_2(S_2CPh)$ complex; Fc-diOH: ferrocifenol.

because of its remarkable efficacy, combined with a long half-life, but was also accompanied by severe side-effects due to ocular, dermatologic, gastrointestinal, neurological, cardiovascular, thyroid, and pulmonary toxicity (Naccarelli et al., 1985). Nanocapsules of different sizes, loaded with amiodarone in their oily phase were analysed, mainly in terms of drug-loading and *in vitro* release profiles, and were compared to PLGA polymer nanoparticles. Interestingly, with the same drug content, the PLGA nanoparticles quickly released amiodarone, with a marked burst effect, whereas the LNCs showed only a small initial burst effect followed by slow sustained release of the encapsulated drug (Fig. 4). This controlled release might essentially result from lipophilic and amphiphilic properties that allow amiodarone to settle at the oil–water interface of formulated LNCs. This can be of interest in the field of drug delivery for topical or systemic application, and also in the domain of cosmetics.

Consequently, many lipophilic drugs, as well as amphiphilic drugs, have been prepared in LNC form as, for example, ibuprofen, incorporated into LNCs for pain treatment by intravenous administration (Lamprecht et al., 2004); indinavir, an inhibitor of HIV-1 protease (Pereira de Oliveira et al., 2005); various hydrophobic anticancer agents: etoposide (Lamprecht and Benoit, 2006), paclitaxel (Lacoeuille et al., 2007), tripentone (Malzert-Freon et al., 2006), derivatives of 4-hydroxy tamoxifen combined with ferrocen (Allard et al., 2008b), etc. In addition, LNCs can be loaded with different radionuclides in their oily core or at their surface such as ^{99m}Tc, ^{188}Re, ^{125}I or ^{111}In (Ballot et al., 2006; Jestin et al., 2007; Allard et al., 2008a). These LNCs provide opportunities for their application in imaging and radiotherapy.

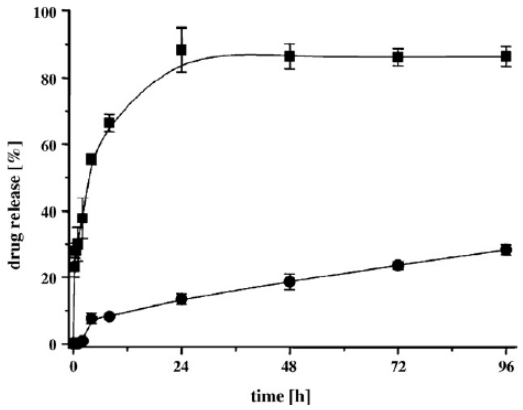

Fig. 4. *In vitro* release profile of amiodarone loaded LNC (circles) and PLGA-NP with blank LNC (squares) as acceptor phase in the release medium (inspired from (Lamprecht et al., 2002)).

3.2. P-glycoprotein inhibition

P-glycoprotein (P-gp), an ATP-dependent drug efflux pump, plays the role of a transporter of various lipophilic and cationic drugs/substrates, preventing sufficient accumulation of anticancer drugs within cells. Indeed, this is one of the main factors leading to the resistance of tumor cells to many anticancer agents

such as anthracyclines (doxorubicin, daunorubicin and epirubicin), *Vinca* alkaloids (vinblastine and vincristine), epipodophyllotoxins (etoposide and teniposide) and taxanes (paclitaxel and docetaxel) (Sparreboom and Nooter, 2000). Therefore, various strategies have been launched in scientific research in order to circumvent this problem (Modok et al., 2006). In fact, several experiments towards the inhibition of P-gp using nanoscale carrier-systems have been carried out. LNCs prepared by the phase-inversion process as described above, demonstrate P-gp inhibiting properties thanks to their ingredients, especially Solutol®. This surfactant is able to block P-gp-related drug efflux with a very low level of *in vitro* toxicity (Coon et al., 1991). Consequently, etoposide-loaded LNCs demonstrate simultaneously P-gp inhibition and sustained drug release from the LNCs (Lamprecht and Benoit, 2006). These adjuvant effects lead to a high level of intracellular, drug accumulation, resulting in increased *in vitro* cytotoxicity on glioma cells.

In this way, the inhibitory effect of multidrug resistance was investigated on an *in vitro* and *in vivo* glioma rat model, treated with either blank LNCs or paclitaxel-loaded LNCs (Garcion et al., 2006). After incorporation into LNCs, paclitaxel showed a potent reduction in 9L and F98 cell survival as compared to a free drug (Taxol®), whereas no significant difference was observed between these two formulations on newborn rat astrocyte primary cells that do not divide. In addition, on a subcutaneous F98 glioma model, paclitaxel-loaded LNC treatment by a single intratumoral injection, significantly reduced tumor mass as well as the evolution of tumor volume (Garcion et al., 2006).

3.3. Passive targeting

Colloidal drug carriers are rapidly removed from systemic circulation after intravenous injection due to their recognition as foreign bodies by the mononuclear phagocyte system (MPS), especially by Kupffer cells in the liver, macrophages in the spleen, and bone-marrow (Moghimi et al., 2001). This recognition is enhanced by the opsonization of the complement system by plasma proteins (Passirani and Benoit, 2005). The elimination of such colloidal systems is influenced by various parameters such as: the nature of the components, their size, the apparent electrical charge, their hydrophilicity (Vonarbourg et al., 2006a). Preferably, they have to be small, composed of natural compounds, and present a neutral and hydrophilic surface. A generation of nanoparticles coated with hydrophilic polymer chains, such as PEG and its derivatives, have been investigated for their property of prolonged circulation time in the bloodstream thanks to steric repulsion generated by the PEG layer (Mosqueira et al., 2001a; Moghimi and Szebeni, 2003).

LNCs appear to satisfy these critical properties because of their nanoscale size range and their shell consisting of a PEG 660 surfactant at high density. ^{188}Re/^{99m}Tc-labelled LNCs exhibited a blood half-life of 21 ± 1 min for ^{99m}Tc and 22 ± 2 min for ^{188}Re in rats (Ballot et al., 2006). Moreover, grafting longer PEG chains, such as PEG 1500 stearate instead of PEG 660 stearate, to the LNC surface provided an opportunity to prolong their circulation residence time (Beduneau et al., 2006). Consequently, the plasma elimination half-life time of these PEG 1500 stearate LNCs has been observed at around 5.5 h, with 20% of total dose still present in the blood 24 h after an intravenous injection into healthy rats. Thus, as well as the high density of PEG at the LNC surface, PEG flexibility linked to the curvature radius and PEG length also play an important role on macrophage uptake (Mosqueira et al., 2001b). These parameters, especially the length of PEG chains, can explain the rapid elimination of conventional LNCs from the blood circulation, despite their very weak complement activation (Ballot et al., 2006; Vonarbourg et al., 2006b).

Furthermore, it has been found to be possible to retain LNCs longer in systemic circulation by post-inserting distearoylphosphatidylethanolamine (DSPE)-PEG 2000 or DSPE-PEG 5000 at their surface with half-life times of over 6 h after intravenous administration (Hoarau et al., 2004). Thus, these pegylated nanocapsules are considered to be potential carriers for drug delivery to the sites of action, particularly into solid tumors due to enhanced permeability and the retention (EPR) effect (Maeda et al., 2000). Indeed, as a consequence of fast growth, tumor vessels are leaky while normal capillaries are found as tight junctions. This vascular defect, coupled with impaired lymphatic drainage, serves to enhance the permeability and retention of nanoparticles within the tumor region, and allow passive targeting to solid tumors using nanocarriers. As expected, docetaxel-loaded LNCs coated with DSPE-PEG 2000 significantly and substantially accumulated in C26 colon adenocarcinoma subcutaneous tumors, whereas uncoated LNCs showed poor tumoral accumulation (Khalid et al., 2006). Indeed, tumoral docetaxel concentrations increased over a 12 h sampling period and were much higher than those of a control docetaxel formulation (Taxotere®).

3.4. Active targeting

Whereas passive targeting takes advantage of a natural, physiological uptake mechanism, active targeting involves the attachment of a homing moiety, such as a monoclonal antibody (MAb) or a ligand, in order to deliver a drug to pathological sites or to cross biological barriers based on molecular recognition processes (Fenart et al., 1999; Brigger et al., 2002; Jallouli et al., 2007). This strategy appears useful for drug delivery into the brain which is limited mainly by the obstacle of the blood–brain barrier (BBB), separating the blood from the cerebral parenchyma (Pardridge, 2005). Consequently, active brain-targeting strategies that have been established to date, consist in grafting a MAb onto a nanocarrier, which can then recognise an over-expressed receptor on brain capillary endothelial cells (Beduneau et al., 2007a). In fact, several studies have investigated the use of OX26 monoclonal antibodies (OX26 MAb) as a vector for drug delivery across the BBB via a receptor-mediated transcytosis mechanism (Friden et al., 1991; Pardridge et al., 1991). This antibody can attach itself directly to the transferrin receptor (TfR) which is selectively localized on the brain capillary endothelium, and is found at low concentrations on other tissues (Jefferies et al., 1984). Moreover, TfR is over-expressed at the surface of proliferating cells such as brain tumor cells, especially on glioblastoma multiforme (Hall, 1991).

In this context, immuno-nanocapsules have been designed, by the conjugation of LNCs to whole OX26 MAb, for the purpose of actively transporting drugs to the brain parenchyma (Beduneau et al., 2007b). Furthermore, Fab′ fragments conjugated to LNCs have also been evaluated because of the interest of their reduced MPS uptake via the Fc receptor-mediated mechanism, which allows prolonged systemic circulation (Maruyama et al., 1997). This coupling has been facilitated by the incorporation of lipid PEG 2000, functionalized with reactive maleimide groups (DSPE–PEG 2000–maleimide), into LNC shells by a post-insertion procedure allowing the covalent attachment of the ligands to LNCs. The OX26 MAb and Fab′ elements were previously thiolated to enable them to react with the reactive maleimide group via thioether bonds. Except in cases where large amounts of antibodies are grafted onto the LNCs, in which case the sizes become heterogeneous, the obtained immuno-nanocapsule size ranged around 150 nm in diameter with a coupling efficiency comprised between 20 and 29%. On the other hand, a size increase after ligand conjugation moved the location of OX26 MAb and Fab′ to outside the PEG brush which facilitated cell association. Further research has included *in vitro* studies on cells over-expressing TfR, such as the Y3.AG.1.2.3. hybridoma cell line

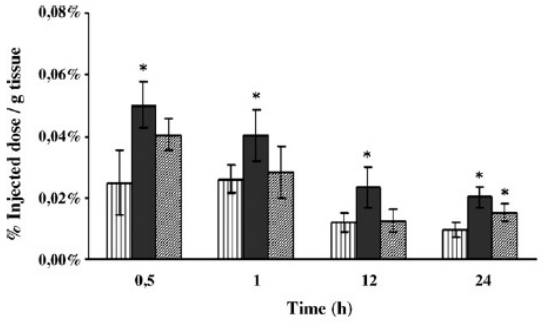

Fig. 5. Brain concentration (% injected dose/g tissue) of functionalized nanocapsules (vertically dashed columns), OX26-immuno-nanocapsules (closed columns) and Fab′-immuno-nanocapsules (right-dashed columns) at different times. Values represent means ± SEM ($n = 5$). *Statistically significant differences to functionalized nanocapsules (Mann–Whitney), $p < 0.05$ (inspired from (Beduneau et al., 2007a)).

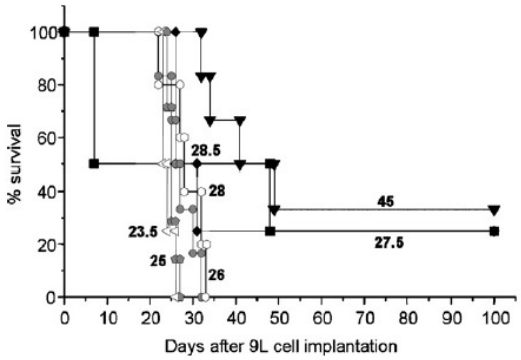

Fig. 6. Kaplan–Meier survival curve of different animal groups treated on Day 6 with 12 Gy ^{188}Re-SSS LNC ($n = 4$; black squares), 10 Gy ^{188}Re-SSS LNC ($n = 4$; black diamonds), 8 Gy ^{188}Re-SSS LNC ($n = 6$; black triangles), 3 Gy ^{188}Re-SSS LNC ($n = 5$; blank hexagons), 4 Gy ^{188}Re perrhenate ($n = 4$; blank triangles), blank LNC ($n = 7$; grey circles) or not treated ($n = 8$; grey pentagons). Numbers represent the median survival time of corresponding group (inspired from (Allard et al., 2008a)).

and rat brain cerebral endothelial cells (BCECs), as well as immuno-nanocapsule distribution in healthy rats after intravenous injection (Beduneau et al., 2008). Ligand density per immuno-nanocapsule has been adjusted between 30 and 40 OX26 MAb or Fab′ fragments. Experimental results from flow cytometry elicited the binding of immuno-nanocapsules to cells via TfR. Concerning biodistribution, as expected, Fab′-immuno-nanocapsules were retained longer in the bloodstream in comparison with OX26-immuno-nanocapsules which are quickly absorbed by the liver. On the contrary, brain accumulation of OX26-immuno-nanocapsules was more efficient than Fab′-immuno-nanocapsules (Fig. 5). However, both types of immuno-nanocapsules provided significant accumulation in the brain 24 h after administration when compared to non-targeted LNCs (2- and 1.5-fold higher respectively) (Beduneau et al., 2008).

Such immuno-nanocapsules represent promising nanocarriers for the active targeting of drug delivery to brain tumors. Further studies on brain tumor-bearing animal models, as well as drug encapsulation into immuno-nanocapsules, might be required to elucidate their targeting possibilities and their potential application in cancer treatment.

3.5. Local treatment

As mentioned above, the delivery of drugs across the BBB is very limited, leading to the failure of major conventional systemic chemotherapies in brain cancer. This is particularly the case of glioblastoma multiforme, this being the most prevalent of malignant glioma in adults, resulting in highly unfavorable survival prognoses (Ohgaki and Kleihues, 2005). Direct intracranial drug delivery by stereotaxic injection would eliminate the need for a chemotherapeutic agent to bypass the BBB. Among the different injection strategies, convection-enhanced delivery (CED) (Vogelbaum, 2005), using an external pressure gradient inducing fluid convection in the brain via a surgically implanted catheter, allows greater volume distribution to be achieved in comparison to diffusion alone (Bobo et al., 1994). Therefore, a combination of nanotechnology with the CED technique appears promising as an approach for direct drug delivery for the treatment of brain tumors (Sawyer et al., 2006; Allard et al., 2009).

In this way, local treatment with the ^{188}Re-SSS complex (^{188}Re(S_3CPh)$_2$(S_2CPh))-loaded LNCs by means of CED on a 9L intracranial xenograft model of a rat brain tumor has been investigated (Allard et al., 2008a). The different doses of ^{188}Re (12, 10, 8 and 3 Gy) were evaluated based on animal survival time. Interestingly, the 8 Gy ^{188}Re-SSS LNC-treated group showed a significant improvement in median survival time as compared to a control group and a blank LNC-treated group (Fig. 6). The increase in the median survival time was about 80% compared to the control group, and 33% of the animals were long-term survivors (over 100 days). The dose of 8 Gy proved to be effective, between toxic (10–12 Gy) and ineffective (3–4 Gy) doses. In addition, this formulation of LNCs was eliminated more slowly than the classical solution of ^{188}Re (perrhenate $^{188}ReO_4^-$) which is recovered very quickly in the urine. LNCs ensured a prolonged therapeutic effect and can be considered as a promising radio-pharmaceutical carrier for internal radiotherapy of brain tumors.

The same method was recently used for the local delivery of LNCs loaded with ferrocenyl diphenol molecules called 'ferrociphenol' (Fc-diOH) followed by external beam irradiation on a rat brain tumor model (Allard et al., submitted for publication). The results showed a synergistic antiproliferative effect between Fc-diOH–LNCs and *in vitro* radiotherapy on 9L glioma cells as well as in an intracerebral *in vivo* 9L glioma model. Previously, these Fc-diOH–LNCs were shown to be cytotoxic on 9L glioma cells ($IC_{50} = 0.6$ μM) and harmless on healthy brain cells up to a concentration range of 10 μM (Allard et al., 2008b). Moreover, in the same study, an antitumor effect was also obtained after a single intratumoral injection at Day 6 after subcutaneous 9L injection on Fischer F344 rats where Fc-diOH–LNCs treatment dramatically reduced both tumor mass and tumor volume.

3.6. Oral administration

Oral drug administration remains the preferred route of administration to ensure patient satisfaction and compliance because it is easy to handle and does not require medical assistance or equipment, and thereby does not necessitate hospitalization. However, it is evident that not all drugs can be administered orally due to the influence of various factors on their pharmacokinetic profile, such as physico-chemical properties, pharmaceutical factors, and physiological factors of the gastrointestinal system (Undevia et al., 2005). The presence of P-glycoprotein (P-gp) on the enterocyte surface limits oral bioavailability (Oostendorp et al., 2009).

As mentioned previously, paclitaxel is pumped out by P-gp which also limits the oral uptake of paclitaxel and mediates the direct excretion of the drug from the systemic circulation into the intestinal lumen (Sparreboom et al., 1997). Therefore, the strategy to enhance the bioavailability of paclitaxel by oral administration is very interesting and requires novel formulations. This issue can

also be addressed by the entrapment of drug molecules in the LNCs (Peltier et al., 2006). Indeed, by loading paclitaxel into the oily core of LNCs, its mean plasmatic concentration is 3 times higher compared to the conventional formulation (Taxol®) and is 1.5 times higher than the co-administration of Taxol® and verapamil, which is known as a major P-gp inhibitor.

Thus, such drug/carrier particulate systems provide an attractive and exciting drug delivery approach for highly potent drug substances that are usually unsuitable for oral use. Further studies will be carried out to elucidate the benefits of this approach compared to conventional drug formulations, their compatibility for large-scale industrial production, and the stringency of registration requirements (Wawrezinieck et al., 2008).

4. Conclusion

Lipid nanocapsules provide a new tool which contributes to nanomedicine development. Using FDA-approved constituents, LNCs are prepared by a solvent-free process to obtain particles of less than 100 nm with monodispersity. These LNCs provide considerable drug encapsulation capacity and also exhibit sustained-release functions at the site of action. Moreover, thanks to the polyoxyethylene-type nonionic surfactant surface, LNCs display a P-gp inhibitory effect harmonized with a stealth effect versus the complement system as well as MPS uptake; they also can be grafted with ligands for the purpose of actively targeting drug delivery.

The interesting possibilities cited in this article for the use of LNCs, allow their use in many therapeutic applications, not only for drug delivery, cancer diagnosis and therapy, but also for gene and cell therapy. Consequently, a generation of LNCs loaded with nucleic acid was recently investigated and patented (Table 1). This opened up an opportunity for the development of gene therapy via a non-viral vector, instead of viral vectors which present many limitations concerning safety, immunogenicity, low transgene size and high costs (Morille et al., 2008). In fact, hydrophilic DNA molecules can be encapsulated into the oily core of LNCs leading to the formation of neutral, 110-nm DNA nanocapsules (Vonarbourg et al., 2009). With the goal of increasing systemic delivery by making DNA LNCs stealthy, DNA LNCs were coated with amphiphilic and flexible PEG polymers [PEG lipid derivative (DSPE-mPEG 2000) or F108 poloxamer] (Morille et al., in 2009). Furthermore, to overcome the internalization difficulties encountered with PEG shields, active targeting using galactose has also been designed for the purpose of efficient hepatocyte targeting (Morille et al., in 2009).

Therefore, LNCs are also adequate systems to encapsulate lipophilic complexes of hydrophilic molecules. Nevertheless, an alternative could be the encapsulation of free hydrophilic molecules inside an aqueous core. New hydrophilic drug delivery systems have been developed in this way (see Table 1).

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Publication de résultats n°1

Effet de la dose de FcdiOH sur un modèle orthotopique de gliome 9L chez le rat

Ce chapitre constitue une transition avec les travaux préliminaires réalisés sur le produit FcdiOH et deux de ses prodrogues (Fc-diAC et Fc-diPAL). En ce qui concerne la molécule FcdiOH, sur laquelle va porter la suite des travaux, les résultats décrivent l'influence de la dose testée dans un modèle orthotopique de gliosarcome 9L chez le rat. Dans cette optique, une étude de survie est mise en place pour évaluer l'efficacité du traitement variant soit avec la quantité de FcdiOH encapsulée au sein des LNC, soit avec le volume d'injection chez l'animal. Différentes doses de FcdiOH (0.005, 0.036 et 2.5 mg de FcdiOH par animal) sont administrées localement par simple stéréotaxie (10µL) ou par convection-enhanced delivery (CED) (60µL), 6 jours après l'implantation des cellules 9L. Le taux de charge du FcdiOH dans le cœur des LNC est soit de 1 mg par gramme de suspension, soit de 6.5 mg/g lorsque le volume d'eau de trempe est réduit de 12.5 mL à 2 mL (de 70% à 28.5% v/v). Par ailleurs, afin de maximiser la dose administrée, le FcdiOH est également infusé à sa solubilisation limite dans le Labrafac® (40 mg/g) constitutif du cœur des LNC. Les 3 groupes d'animaux ayant reçu ces différentes doses sont comparés à des rats traités par une suspension de LNC blanches, par le Labrafac® seul ou à des rats non traités.

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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 anticancer 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 μM while Fc-diOH-LNCs were harmless on healthy brain cells up to a concentration range of 10 μM (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

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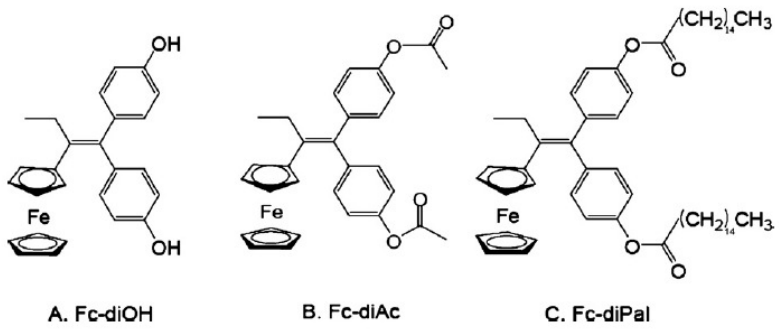

Fig. 1. Chemical formulae of ferrocifen molecules; 2-ferrocenyl-1,1-bis(4-hydroxyphenyl)-but-1-ene called Fc-diOH (MMr = 424.4 g mol^{-1}) and its prodrugs Fc-diAc (MMr = 508.4 g mol^{-1}) and Fc-diPal (MMr = 901.4 g mol^{-1}).

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.

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, CH_2Cl_2 was added, and the mixture was decanted. The aqueous phase was extracted with CH_2Cl_2, the combination of organic layers was dried under magnesium sulphate, concentrated under reduced pressure, and chromatographed with CH_2Cl_2. For Fc-diPal: Yield 91%. Anal. Calc. for $C_{58}H_{84}FeO_4$: 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). Lipoïd® 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 Lipoïd 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®, Vétoquinol, 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 μL) 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).

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

2.5.3. An orthotopic xenograft model

For intracranial implantation, 10 μL of 10^3 9L cell suspension were injected into the rat striatum at a flow rate of 2 μL/min using a 10 μL 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 μL) or by convection-enhanced delivery (CED) (infusion volume = 60 μL) (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 μL 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 ± 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 nanopartic-

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 1 mg/g	51.3 ± 0.6	0.093 ± 0.006	−11.1 ± 1.6	98.1 ± 1.7
Fc-diAc LNCs 1.2 mg/g	54.2 ± 2.9	0.084 ± 0.020	−14.0 ± 6.3	96.2 ± 2.9
Fc-diPal LNCs 2.1 mg/g	60.1 ± 0.3	0.125 ± 0.006	−17.4 ± 6.7	97.9 ± 0.5

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

ulate 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.

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 μmol/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 μmol/L) and near to zero for concentrations above 10 μmol/L, with an IC_{50} value of about 0.6 μmol/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 (Bartosz, 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 μmol/L for Fc-diPal-LNCs whereas the incubation of blank LNCs at the same excipient concentration gave IC_{50} values of around 60 μmol/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.

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 mm^3, 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

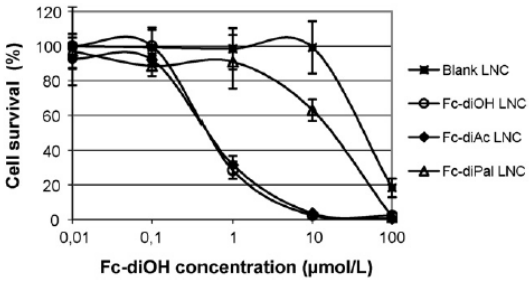

Fig. 2. Cell survival test of 9L cells after 96 h of treatment with ferrocifen-loaded LNCs at concentrations from 0.01 to 100 μmol/L equivalent Fc-diOH. Blank LNCs are tested with the same excipient concentrations as for Fc-diOH-loaded LNCs. Blank LNCs, Fc-diOH-LNCs 1 mg/g, Fc-diAc-LNCs 1.2 mg/g and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

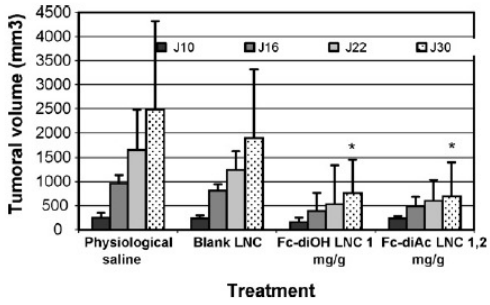

Fig. 3. *In vivo* effects of Fc-diOH and Fc-diAc-loaded LNC treatment on the growth of 9L-glioma cells implanted subcutaneously on Fisher rats. Efficacy of the treatments with ferrocifen-loaded LNCs was compared with controls made after a single injection of physiological serum or blank LNCs. The graph represents an estimation of tumour growth assessed by tumour size measurements ± 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.

volume increased from 240 ± 60 to 1900 ± 1420 mm^3 between Day 10 and Day 30 for the animals treated with a single injection of blank LNCs and from 250 ± 100 to 2500 ± 1830 mm^3 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 mm^3 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 mm^3 (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®.

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

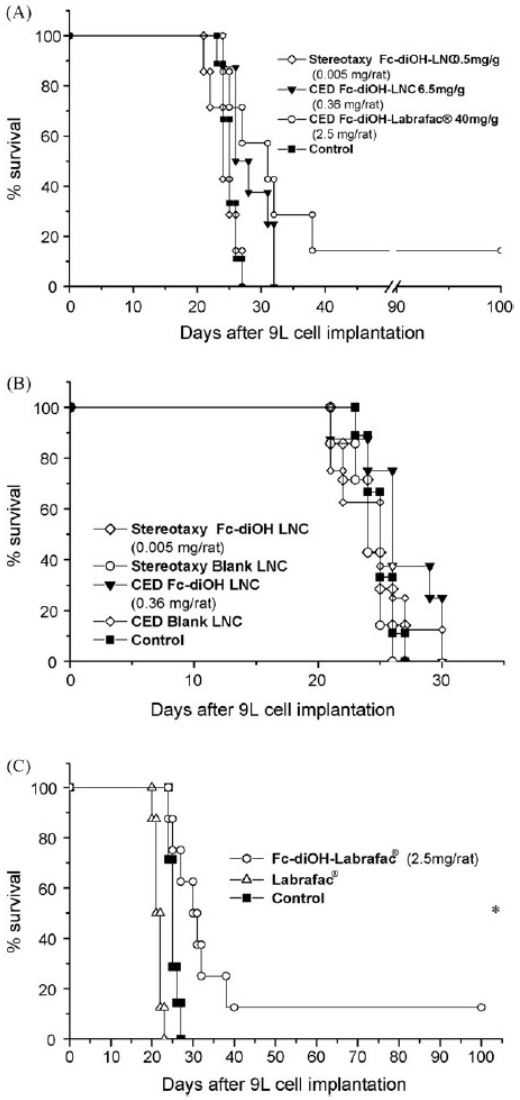

Fig. 4. Kaplan–Meier survival curves for 9L-glioma bearing rats for escalating doses of Fc-diOH encapsulated in LNCs or solubilised in Labrafac® versus appropriate controls. (A) represents the survival curves in days after tumour implantation for untreated animals = control group (-■-), stereotaxy of 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).

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

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

Treatment	Injection	n	Survival time (days)			Increase survival time (%)		
			Range	Median	Mean ± 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 (%).

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.

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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Publication de résultats n°2

Influence de la voie d'administration des LNC-FcdiOH sur l'efficacité du traitement des gliomes

Les études précédentes ont mis en évidence des séquelles neurologiques au sein de l'hémisphère injecté des rats longs survivants, porteurs d'un gliosarcome intracérébral, ayant reçu des suspensions concentrées de LNC-FcdiOH à 6.5 mg/g. La spectroscopie de résonance magnétique nucléaire montre une décroissance importante du taux de N-acétyl-aspartate (NAA) et de créatine, ce qui témoigne d'une souffrance cellulaire avérée. Ces séquelles neurologiques peuvent être la conséquence d'une hyper-osmolarité des formulations administrées. En effet, l'osmolarité de la suspension concentrée est plus de deux fois supérieure à celle des conditions physiologiques (760 mmol/kg par rapport à 300 mmol/kg).
L'objectif de ce travail est d'évaluer les effets secondaires par imagerie et spectroscopie à résonance magnétique de l'administration de la suspension hyperosmolaire de LNC-FcdiOH par CED dans le striatum des rats sains. Après ajustement aux valeurs physiologiques, l'efficacité antitumorale de ces suspensions est évaluée. Par ailleurs, l'effet cytostatique du FcdiOH étant proportionnel à la dose administrée par cette voie (publication n°1), l'administration intra-carotidienne des LNC-FcdiOH, qui permet d'augmenter le volume injecté, est également étudiée. Cette voie d'administration est connue pour être efficace vis-à-vis de la délivrance des principes actifs dans le cerveau. Dans un but de bénéficier des propriétés furtives apportées par le poly(éthylène) glycol (PEG), l'efficacité des LNC recouvertes par de longues chaines de PEG (DSPE-mPEG2000) est comparée à celle des LNC classiques. Enfin, la localisation des différentes sortes de LNC est mise en évidence de manière semi-quantitative sur des coupes de cerveau après marquage des LNC avec un fluorochrome (le DiI).

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Administration-dependent efficacy of ferrociphenol lipid nanocapsules for the treatment of intracranial 9L rat gliosarcoma

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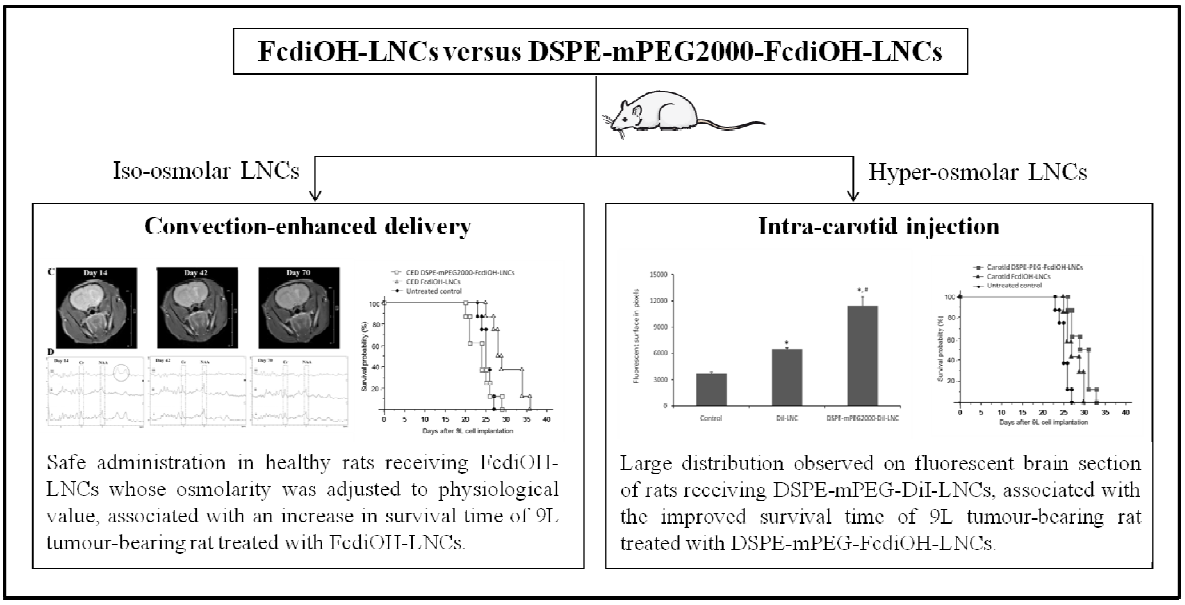
Graphical abstract



Abstract

The anti-tumour effect of ferrociphenol (FcdiOH)-loaded lipid nanocapsules (LNCs), with or without a DSPE-mPEG2000 coating, was evaluated on an orthotopic gliosarcoma model after administration by convection-enhanced delivery (CED) technique or by intra-carotid injection. No toxicity was observed by MRI nor by MRS in healthy rats receiving a CED injection of FcdiOH-LNCs (60µL, 0.36mg of Fc-diOH/rat) when the pH and osmolarity had been adjusted to physiological values prior to injection. At this dose, the treatment by CED with FcdiOH-LNCs significantly increased the survival time of tumour-bearing rats in comparison with an untreated group (28.5 days vs 25 days, P = 0.0009) whereas DSPE-mPEG2000-FcdiOH-LNCs did not exhibit any efficacy with a median survival time of 24 days. After intra-carotid injection (400µL, 2.4mg of FcdiOH/rat), hyperosmolar DSPE-mPEG2000-FcdiOH-LNCs markedly increased the median survival time (up to 30 days, P = 0.0008) as compared to the control (20%). This was strengthened by their evidenced accumulation in the tumour zone and by the measure of the fluorescent brain surface obtained on brain slides for these DiI-labelled LNCs, being 3-fold higher than for the control. These results demonstrated that, depending upon the administration route used, the characteristics of LNC suspensions had to be carefully adapted.

Keywords: brain tumour, DSPE-mPEG2000, convection-enhanced delivery (CED), intra-carotid injection, PEGylated nanoparticles.

1. Introduction

The incidence rate of brain tumours has increased remarkably and tended to be higher in developed, industrialised countries (Parkin *et al.*, 2005). Among the various types of brain tumour, glioblastomas represent the most prevalent and malignant gliomas in adults (grade IV classified by the World Health Organization - WHO) (Louis *et al.*, 2007). In spite of numerous, notable advances in diagnosis techniques and also in multimodal therapy regimes including surgical resection followed by radiotherapy and adjuvant chemotherapy (Stupp *et al.,* 2005), the survival prognosis always remains unfavourable, most patients dying within 2 years after diagnosis (Grossman and Batara, 2004). In terms of chemotherapy, the effort in drug discovery and development has been limited due to the obstacle of the blood-brain barrier (BBB) which prevents the delivery of drugs to the central nervous system (CNS) by means of a systemic injection (Pardridge, 2005; Segal, 2000).

Over the last decades, the technique of drug delivery to the brain by convection-enhanced delivery (CED) has provided an interesting tool of administration for brain cancer chemotherapy (Bobo *et al.*, 1994). This technique consists in the continuous injection of a therapeutic fluid under positive pressure. CED injection allows a local administration of large doses of infusate which is mainly distributed within the interstitial spaces of tissues by a gradient of pressure that enhances constitutive intracellular medium convection (Allard *et al.*, 2009b). Indeed, by using lipid nanocapsules (LNCs) which are characterised by a hybrid structure between polymer nanocapsules and liposomes (Huynh *et al.*, 2009), a previous study demonstrated the wide distribution area throughout the injected hemisphere (Vinchon-Petit *et al.*, 2010). Homogeneity of Nile Red-labelled LNCs around the injection site was observed for 24h following a CED injection into the brain of healthy rats. Moreover, on 6-day-old 9L tumour-bearing rats, Nile Red-labelled LNCs fully covered the tumour zone and were found in the cytoplasm of tumour cells.

Recently, a new series of organometallic tamoxifen derivatives have been developed by adding a potentially cytostatic ferrocene moiety to the tamoxifen skeleton (Jaouen *et al.*, 2000a; Top *et al.*, 2003; Vessieres *et al.*, 2005). The mechanism of these ferrocene derivatives is believed to act as an intramolecular catalyst for the oxidation of the phenol group in mild conditions, leading to the production of quinone methides which are strongly toxic (Hillard *et al.*, 2005). These last compounds may interact with macromolecules such as glutathione, DNA and certain proteins leading to the tumour cell's death by accelerated senescence (Vessieres *et al.*, 2005). Among these derivatives, the compound 2-ferrocenyl-1,1-bis(4-hydroxyphenyl)-but-1-ene, or ferrociphenol compound (FcdiOH) has proved *in vitro* to be an effective cytostatic compound (IC_{50} = 0.7µM on MCF-7 hormone-dependent and IC_{50} = 0.44µM on MDA-MB231 hormone-independent breast cancer cell lines) (Vessieres *et al.*, 2005). FcdiOH loaded into the oily core of LNCs iOH-LNCs at high concentrations (6.5mg/g, 2% dried weight) showed remarkable antitumour effects in a subcutaneous, 9L gliosarcoma model after an intra-tumoural injection (Allard *et al.*, 2008b). FcdiOH-LNCs improved the survival time of rats in an intracranially-implanted 9L gliosarcoma model when administered by CED in a dose-dependent manner (Allard *et al.*, 2009a). Moreover, a synergistic effect was also observed between chemotherapy with FcdiOH-LNCs and external irradiation revealing the radio-sensitive properties of FcdiOH (Allard *et al.*, 2010). However, some neuronal side effects in the injected hemisphere observed in long-term survivors receiving the highest LNC suspension concentration have been reported previously (Allard *et al.*, 2008a).

This study aimed at evaluating the impact of FcdiOH-LNCs on the rat brain after CED administration by magnetic resonance imaging before and after some adjustments assessed as needed for effective and safe administration. Furthermore, the antitumour efficacy of these modified FcdiOH-LNCs delivered by CED technique on an intracranial gliosarcoma rat

model was evaluated. As an alternative route of drug administration to the brain (Alam *et al.*, 2010), intra-carotid injection of FcdiOH-LNCs through the carotid artery was investigated in order to increase the volume of drug administered. Moreover, the efficacy of FcdiOH-LNCs coated with longer chains of polyethylene glycol (PEG) was also assessed in order to test the effect of long-circulating properties brought by this polymer (Morille *et al.*, 2010).

2. Materials and methods

2.1. Materials

Ferrociphenol, abbreviated as FcdiOH, was prepared by a McMurry coupling reaction (Jaouen *et al.*, 2000b). The lipophilic Labrafac® CC (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). 1,2-DiStearoyl-sn-glycero-3-Phospho-Ethanolamine-N-[methoxy-(polyethylene glycol)-2000] (DSPE-mPEG2000) (Mean Molecular Weight (MMW) = 2805g/mol) was kindly provided by Avanti Polar Lipids (Alabaster, USA). 1,10-dioctadecyl-3,3,30,30-tetramethylindocarbocyanine perchlorate (DiI) was obtained from Introgen (Cergy, Pontoise, France).

2.2. Preparation of the LNCs

2.2.1. Anticancer agent-loaded LNCs

In this study, fifty nanometer-diameter LNCs, whose formulation is based on the phase-inversion phenomenon of a microemulsion, were prepared in conformity with the described

procedure (Heurtault *et al.*, 2002). Briefly, the preparation process involved 2 steps. Step I consisted of mixing all the components (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)) 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 (70% v/v) to the mixture at 70-72°C. Slow magnetic stirring was then applied to the suspension for 5 minutes.

To load the anticancer agent to the oily core of LNCs, FcdiOH was firstly dissolved in Labrafac® under ultrasound at 4% (w/w) for 1.5 hours and the resulting lipophilic phase was afterwards mixed with other components as described above. Moreover, in this study, in order to increase the concentration of the drug in the final LNC suspension, the cold water added to the mixture at the step II was decreased from 70% (v/v) for conventional formulations (111mg of particles per gram of suspension) to 28.5% (v/v) for the concentrated ones (280 mg/g). Final LNC suspensions were filtered through a Minisart 0.2µm filter (Sartorius) in order to eliminate some unincorporated products.

2.2.2. Fluorescent LNCs

To visualise the LNCs on the brain slides, LNCs were labelled with DiI (emission wavelength = 549nm; excitation wavelength = 565nm). The formulation of fluorescent LNCs was similar to that described previously (Garcion *et al.*, 2006). Briefly, DiI was dissolved in acetone at 3mg/mL and the resulting solution was incorporated in the Labrafac® at proportion of 1:30 (v/v). Acetone solvent was then evaporated before mixing with other components to formulate fluorescent LNCs.

2.2.3. Surface-modified LNCs

DSPE-mPEG2000 was incorporated to the surface of LNCs at the concentration of 10mM by the post-insertion technique previously described (Morille *et al.*, 2010). Briefly, preformed LNC suspensions and DSPE-mPEG2000 micelles were co-incubated for 2h at 60^0C. The mixture was vortexed every 15 min. and then quenched in an ice bath for 1 minute.

2.2.4. Adjustment of the pH and osmolarity of the LNC suspensions

In certain experimental procedure of CED injection, the concentrated LNC suspensions (referred to as crude suspensions) were passed through a PD-10 sephadex column (Amersham Biosciences Europe, Orsay, France) and then concentrated by ultra-filtration with Millipore Amicon 100kDa centrifugal filter device (Millipore, St Quentin-Yvelines, France). The osmolarity of the resulting suspensions was then adjusted by adding a 5M saline solution. Finally, the acidity was neutralised by a 0.1N NaOH solution in order to obtain adjusted LNCs.

2.3. Characterisation of LNCs

2.3.1. Mean particle size and zeta potential

The LNCs were diluted 1:100 (v/v) in deionised water and the measurements were performed at 25^0C. The LNCs were analysed in triplicate for their mean particle diameter, polydispersity index (PdI) and surface charge using a Malvern Zetasizer® (Nano Serie DTS 1060, Malvern Instruments S.A., Worcestershire, UK).

2.3.2. Osmolarity and pH measurement

The measurement of the pH and osmolarity of the LNC suspensions was performed by using a Consort C561 pH meter from Avantec (Fisher Bioblock, Scientific) and 5520 Vapro vapour osmometer from Wescor (Logan, Utah, USA), respectively.

2.4. *In vivo* studies

2.4.1. Animals

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

2.4.2. Tumour cell culture

Rat 9L gliosarcoma cells were obtained from the European Collection of Cell Culture (Salisbury, UK, N°94110705). The cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) with glucose and L-glutamine (BioWhittaker, Verviers, Belgium) supplied with 10% foetal calf serum (FCS) (BioWhittaker) and 1% antibiotic and antimycotic solution (Sigma, Saint-Quentin Fallavier, France).

2.4.3. Intra-cranial gliosarcoma model

On the day of implantation, a cultured tumour monolayer was detached by using a mixture of trypsin-ethylene diamine tetraacetic acid, then, washed twice with Eagle's minimal essential medium (EMEM) without FCS and antibiotics. Cells were counted, and re-suspended in EMEM to the final concentration of 10^5 9L cells/mL for implantation.

The animals were anaesthetised by intra-peritoneal injection of 1.0ml/kg of a 1:1 mixture of ketamine (100mg/ml) (Clorketam®, Vétoquinol, Lure, France) and xylazine (20mg/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. The cannula coordinates were 1mm posterior from the bregma, 3mm lateral from the

sagittal suture, and 5mm below the dura (with the incisor bar set at 0mm). The 32G needle (Hamilton®), fitted to a 10µl syringe (Hamilton® glass syringe 700 series RN), was left in place for 5 minutes and then 10 microlitres of 10^3 9L cell suspension were injected stereotaxically into the right rat striatum at a flow rate of 2µl per minute. 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 (0.5mm/minute). The scalp incision was then closed by using a silk suture (Perma-Hand Seide 3-0, Ethicon).

On Day 6 after 9L cell implantation, rats were injected by means of CED or intra-carotid injection of different LNC formulations.

2.4.4. CED procedure

Tumour-bearing-rats were anaesthetised by an intra-peritoneal injection of 1.5ml/kg of a 2:1 mixture of ketamine and xylazine. The same procedure of injection as described for cell implantation was applied. The 32G needle (Hamilton®) fitted to a 10µl Hamilton® syringe was placed in the same coordinates. This syringe was connected to a 100µl Hamilton syringe 22G containing the product (Harvard Apparatus, Les Ulis, France) through a cannula (CoExTM PE/PVC tubing, Harvard apparatus, Les Ulis, France). Sixty microlitres (60µL) of LNC suspension were injected by CED which was performed with a pump PHD 2,000 infusion (Harvard Apparatus, Les Ulis, France) by controlling a 0.5µl/min rate for two hours. 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 (0.5mm per minute).

2.4.5. Intra-carotid injection

Tumour-bearing-rats were anaesthetised by an intra-peritoneal injection of 1.0-1.5ml/kg of a 1:1 solution of ketamine (100mg/ml) (Clorketam®, Vétoquinol, Lure, France) and xylazine

(20mg/ml) (Rompun®, Bayer, Puteaux, France). The middle neck skin was shaved and incised. The right common carotid (the same side of 9L cell implantation) was exposed and ligated. A PE10 polyethylene catheter (BD Intramedic™ Polyethylene Tubing, Becton Dickinson, USA) was inserted retrogradely through a small arteriotomy. 400µL of LNC suspension bolus were injected in into each rat. The catheter was then removed and the carotid artery was ligated.

2.4.6. Side effect monitoring by magnetic resonance imaging and ^{1}H-magnetic resonance spectroscopy

60µL FcdiOH-LNCs were administered by CED (as described in section 2.4.4) to healthy rats at the same coordinates of tumour implantation. Magnetic resonance imaging (MRI) and ^{1}H magnetic resonance spectroscopy (MRS) were taken on Days 14, 42, 70 and Day 100 after CED injection, to monitor side effects of crude suspension and adjusted suspension of FcdiOH-LNCs with physiological osmolarity and pH values. MRI was performed on a Bruker Avance DRX 300 equipped with a vertical superwide-bore magnet operating at 7T. Rapid qualitative T2-weighted images were obtained using rapid acquisition with relaxation enhancement (RARE) sequence (TR = 2,000ms; mean echo time (TE) = 31.7ms; RARE factor = 8; FOV = 3 × 3cm; matrix 128 × 128; nine contiguous slices of 1mm, eight acquisitions). ^{1}H MRS was performed using a PRESS sequence with water suppression under the following parameters: TR/TE = 1,500/11ms; NEX = 128; voxel size 27µl (3 ×3 × 3mm), as already determined (Lemaire *et al.*, 1999).

2.4.7. Visualisation of fluorescent LNCs in rat brain

For the purpose of tracking the injected LNCs, they were loaded with a fluorescent agent (DiI) instead of an anticancer agent (FcdiOH). 400µL of DiI-LNCs or DSPE-mPEG2000-DiI-LNCs were administered into tumour-bearing rats through the right common carotid artery.

Untreated tumour-bearing rats served as the control group. The rats were euthanised 24h after injection in a CO_2 chamber. The brain was removed and then snap-frozen in liquid nitrogen-chilled isopentane and stored at -80^{0}C. Coronal cryosections (14µm) throughout the tumour zone were performed and recovered on slides. The slides were kept at least 24h at -20^{0}C before being processed.

Frozen sections were defrosted at room temperature and rehydrated in Dulbecco's phosphate-buffered saline (DPBS). Sections were fixed in 4% paraformaldehyde and washed with DPBS. Slides were observed under an Axioskop-2 Zeiss fluorescent microscope (Le Peck, France) using a 20x objective. The acquired images were analysed by measuring the fluorescent surface area on the brain tissue slides via integrated morphometry analysis of an inclusive threshold image by use of the Metamorph® software (Roper Scientific, Evry, France). In order to better visualise the nucleus of the tumour cells, some sections were further stained with 1/1000 4′,6-diamidino-2-phenylindole (DAPI) in DPBS for 10 min. and then washed with DPBS before mounting.

2.4.8. Efficacy study

The treatment was assessed by CED administration (60µL, 0.36mg of FcdiOH/rat), or intra-carotid injection (400µL, 2.4mg of FcdiOH /rat) with FcdiOH-LNCs or DSPE-mPEG2000-FcdiOH-LNCs. For CED injection, the osmolarity and pH of the LNC suspensions were adjusted to physiological values whereas the crude suspensions were used for intra-carotid injection. The untreated control group did not receive any treatment. Animals were weighed every 6 days. The animals were sacrificed in a CO_2 chamber when they lost 20 % of body weight and/or as soon as they presented seizure, a hunched posture, or haemorrhaging around the eyes, mouth and nose. 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.

The statistical analysis was 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 $P < 0.05$. The different treatment groups were compared in terms of median and mean survival time (days). The percentage of increase in survival time (% IST) was determined relative to the median and mean survival times of the untreated control group as presented in the following equation:

$$\% \text{ IST} = [\text{Median}_T (\text{Mean}_T) - \text{Median}_C (\text{Mean}_C)]/ \text{Median}_C (\text{Mean}_C)$$

where $\text{Median}_T/\text{Mean}_T$ was the median/mean of survival time of treated group while $\text{Median}_C/\text{Mean}_C$ was the median/mean of survival time of control group.

3. Results and discussion

3.1. Physicochemical properties of LNC suspensions

The physicochemical properties of different types of LNCs are presented in Table 1. All types of LNCs had their average size ranging 44 – 53nm with very narrow size dispersion (PdI < 0.08). LNCs were characterised by a slightly negative zeta potential (from -4 to -5mV), except for DSPE-mPEG2000-FcdiOH-LNCs that presented a zeta potential of bout -22mV due to the formation of dipoles between PEG molecules and water, as already described (Vonarbourg *et al.*, 2005). The encapsulation of FcdiOH did not affect the average particle size and zeta potential as previously reported (Allard *et al.*, 2008b). This denoted efficient encapsulation of FcdiOH in the oily core of LNCs.

The reduction of cold water added to the final temperature cycle allowed formulating FcdiOH-LNCs at a high drug concentration (6.5mg of FcdiOH per gram of LNC suspension, corresponding to 2% w/w dry weight). This modification did not alter the particle size nor the zeta potential of resulting LNCs compared to the conventional ones (Table 1). However,

concentrated blank LNCs as well as FcdiOH-LNCs were hyperosmolar (780.25 ± 7.80 and 773.50 ± 7.05mmol/kg, respectively) whereas conventional blank LNCs were hypoosmolar (227.50 ± 2.12mmol/kg). With respect to the suspension acidity, all LNC suspensions presented an acid pH level (pH = 5.3 – 5.7) and there was no difference between conventional formulations and concentrated ones. After adjustments concerning osmolarity and pH, measurement of particle size and zeta potential showed no changes in these parameters (data not shown).

Table 1: Physicochemical properties of LNC suspensions.

		Mean particle size (nm)	Poly-dispersity PdI	Zeta potential (mV)
Conventional formulation	Blank LNCs	49.13 ± 2.18	0.026 ± 0.012	-3.38 ± 0.42
Concentrated formulation	Blank LNCs	49.01 ± 2.18	0.029 ± 0.009	-3.82 ± 0.21
	FcdiOH-LNCs	44.10 ± 0.91	0.025 ± 0.007	-5.05 ± 1.61
	DSPE-mPEG2000-FcdiOH-LNCs	49.08 ± 0.15	0.080 ± 0.006	-21.67 ± 1.10
	DiI-LNCs	49.94 ± 0.22	0.040 ± 0.008	-3.28 ± 0.10
	DSPE-mPEG2000-DiI-LNCs	53.22 ± 1.40	0.041 ± 0.009	-22.50 ± 1.11

3.2. Drug delivery by means of the CED technique

3.2.1. Monitoring of side effects by magnetic resonance imaging

In a previous study reporting the treatment of 9L gliosarcoma in rats by CED injection of a concentrated suspension of ^{188}Re-loaded LNCs, the T2-weighted images taken from long-term survivors showed a hyper-intense signal, which was mainly localised in the striatum and persisted until Day 140 of experiments (Allard *et al.*, 2008a). Moreover, ^{1}H MRS showed a decrease in the signal intensity for N-acetylaspartate (NAA) and creatine (Cr). NAA is

considered as a valuable marker of brain injury (Demougeot *et al.*, 2001). A decrease in NAA levels detected from MRS suggests neuronal/axonal loss, or compromised neuronal metabolism (Moffett *et al.*, 2007; Schuff *et al.*, 2006). The authors postulated that this lesion was related to internal radiation injury. Unfortunately, these side effects were also observed in a long-term survivor rat that was treated with concentrated suspensions of FcdiOH-LNCs in a previous study concerning tumour-bearing rats (unpublished data).

Therefore, the first set of experiments in the present study was to investigate whether crude FcdiOH-LNCs altered the neuronal metabolism and caused the side effects on healthy rats after a CED administration. T2-weighted images showed that the injection of such a suspension caused a lesion, highlighted by a hyper-intense signal region on the injected hemisphere until Day 70 (figure 1A) and characterised by a significant decrease in NAA and Cr intensity of the injected hemisphere as compared to the contralateral hemisphere on proton spectra (Figure 1B). On the other hand, two high peaks around 0.9-1.5ppm were observed from the spectrum taken on Day 14 post-injection, which probably corresponded to the peaks of LNC lipids. However, these peaks nearly disappeared by the following reading (Day 42). This underlined the metabolism of LNCs during 6 weeks after injection into the healthy rat striatum. Moreover, these neuronal lesions were also observed in rats receiving blank LNCs with similar hyper-intense signal region on the injected hemisphere on MRI and a considerable decrease in NAA and Cr levels on ^{1}H MRS (figures not shown). Therefore, such side effects could be related to the intrinsic properties of LNC suspensions.

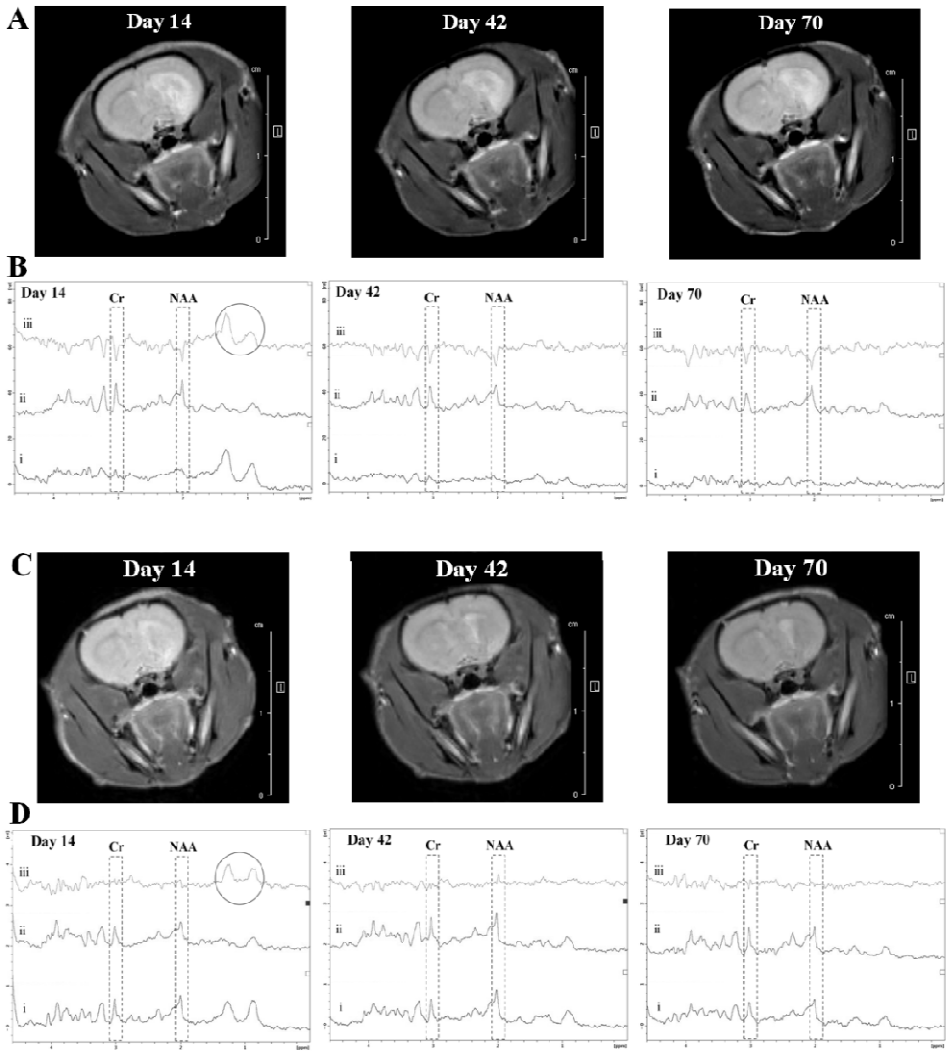


Figure 1: Representative MRI and ^{1}H MRS taken from healthy rats that received crude FcdiOH-LNCs (A-B) and adjusted FcdiOH-LNCs (C-D). CED injection of crude FcdiOH-LNCs caused a lesion on the injected hemisphere as seen on T2-weighted images (A). The lesions were characterised by a decrease in NAA and Cr intensity (i) as compared to the contralateral hemisphere (ii) that was presented by the difference in signal intensity between 2 hemipheres (iii) on the ^{1}H MRS (B). By adjusting the osmolarity and pH of FcdiOH-LNCs to the physiological values, CED injection of adjusted FcdiOH-LNCs showed a limited hyper-intense signal on the injected hemisphere on T2-weighted images (C) and no difference in NAA and Cr intensity was found in ^{1}H MRS between the 2 hemispheres (D). Spectra taken on Day 14 revealed 2 peaks of LNCs (circle) which disappeared on Day 42.

Indeed, administrating a hyperosmotic infusate into the extracellular space produces the movement of water from the intracellular space to the extracellular space, leading to cellular dehydration. This effect potentially induces brain tissue damage, resulting in neurological dysfunction (Petit *et al.*, 2006; Verbalis, 2010). A marked cell volume decrease was observed when incubating cultured human astrocytes with hyperosmotic medium (Anderson *et al.*, 2000). Luh *et al.* demonstrated that the prolonged exposure of endothelial cells to a hyperosmolar medium (> 460mOsm/L) significantly reduced its viability and function (Luh *et al.*, 1996). Therefore, these previously-observed side effects could be linked to the hyperosmolarity of crude LNC suspension which was 2.5 fold higher than physiological osmolarity.

As a consequence, for a safe local administration, we adjusted their osmolarity and pH to physiological conditions as described in part 2.2.4. Effectively, this formulation did not disturb the normal condition of the brain environment since MRI showed a limited hyper-intense signal zone on the injected hemisphere (Figure 1C) associated with no difference in NAA and Cr signal intensity between 2 hemispheres on ^{1}H MRS (Figure 1D).

3.2.2. Efficacy of CED treatment

The survival data from treated tumour-bearing rats are summarised in Table 2 and Kaplan–Meier survival plots are shown in Figure 2. By applying the criteria for euthanasia of experimental animals, rats were sacrificed when they lost 20% of body weight associated with a high degree of depression. All untreated control rats were sacrificed from Day 22 to Day 26 after 9L cell implantation resulting in a median and mean survival time of 25 days.

Table 2: Efficacy of FcdiOH-LNCs and DSPE-mPEG2000-FcdiOH-LNCs in an intracranial 9L gliosarcoma model in Fisher rats (IST: increased survival time).

Administration route (injected dose of FcdiOH)	Treatment	n	Survival time (days)			% IST	
			Range	Median	Mean ± SD	Median	Mean
Convection-enhanced delivery (0.36mg/rat)	FcdiOH-LNCs	8	25-36	28.5	30.13 ± 3.98	14.00	19.90
	DSPE-mPEG2000-FcdiOH-LNCs	8	20-29	24	23.75 ± 3.01	---	---
Intra-carotid (2.4mg/rat)	FcdiOH-LNCs	7	25-30	27	27.57 ± 2.07	8.00	10.29
	DSPE-mPEG2000-FcdiOH-LNCs	8	26-33	30	29.38 ± 2.50	20.00	17.50
	Untreated control	8	23-27	25	25.13 ± 1.25		---

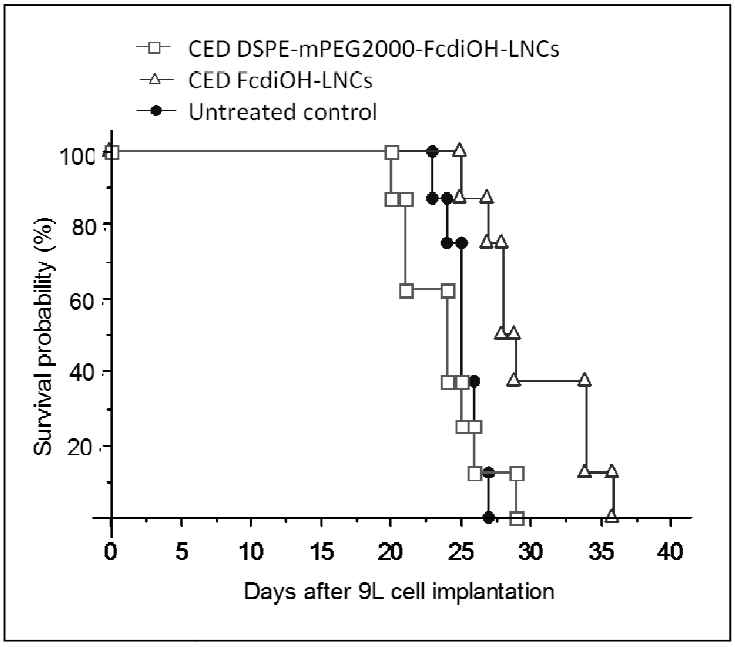


Figure 2: Kaplan–Meier survival plots for 9L glioma-bearing rats receiving CED injection of DSPE-mPEG2000-FcdiOH-LNCs, FcdiOH-LNCs (60µL of LNC suspension, corresponding to 0.36mg of FcdiOH per rat) at Day 6 after cell implantation and untreated animals.

The treatment with adjusted FcdiOH-LNCs (60µL, 0.36mg/rat) by means of CED significantly increased the median and the mean survival time of animals as compared to the control group (28.5 and 30 days, respectively). On the contrary, treatment with DSPE-mPEG2000-FcdiOH-LNCs in the same dose did not improve the survival of animals as their median remained at 24 days.

The failure of DSPE-mPEG2000-FcdiOH-LNCs treatment could be explained by the abundant presence of PEG, especially with the long chains of PEG at the nanoparticulate surface. Indeed, PEG coating potentially inhibits the internalisation of drug-loaded NPs by tumour cells because it is known to prevent the interactions between the NPs and the tumour cell surface (Hong *et al.*, 1999; Morille *et al.*, 2009). Moreover, the moderated efficacy of FcdiOH by CED administration might be linked to the dose of drug used in this study being not enough high. Indeed, as previously observed, FcdiOH exhibited a cytostatic activity *in vivo* on the intracranial 9L gliosarcoma model in rats in a dose-dependent manner (Allard *et al.*, 2009a). In this previous work, the treatment by CED of FcdiOH dissolved in Labrafac (2.4mg/rat) at its limited solubility (40mg/g) gained the median survival time up to 31 days. Unfortunately, some toxic signs were observed in rats treated with drug-free Labrafac showing the limitation of clinical tolerance to Labrafac when infused alone. Thus, taking into consideration the benefit of LNCs in improving intracellular bioavailability (Garcion *et al.*, 2006), these results reveal the necessity of loading FcdiOH into the oily core of LNCs and increasing the volume of the injection as well.

3.3. Drug delivery by intra-carotid injection

Intra-carotid administration can be considered as a regional delivery of drug to the brain that leading to a significant enhancement in drug exposure of the tumoural vasculature (Joshi *et al.*, 2008). In this case, the drug is injected directly into the blood artery and directed towards

the brain vasculature before entering peripheral tissues by avoiding the first pass metabolism (Alam *et al.*, 2010). Extensive studies carried out in both experimental animals as well as in humans, have demonstrated benefits of intra-arterial administration in the treatment of brain tumour (Chertok *et al.*, 2010; Figueiredo *et al.*, 2010; Fortin *et al.*, 2005; Fujiwara *et al.*, 1995; Newton, 2006; Newton *et al.*, 2003; Schem and Krossnes, 1995).

In order to test the benefit of this administration route, LNCs were labelled with DiI for the purpose of tracking the injected LNCs. Moreover, this administration route allowed increasing the volume of injection of LNCs from 60µL for CED injection to 400µL.

3.3.1. LNC visualisation in rat brain slices

Fluorescent section images of control rats that did not receive DiI-LNCs showed the auto-fluorescent spots of brain cells which were considered as background noise, and no fluorescence was detected in the brain capillary lumen (Figure 3A). 24h after administration of labelled LNCs, including DiI-LNCs or DSPE-PEG-DiI-LNCs by carotid injection, red fluorescence of DiI was observed and could be assigned within the brain tissue (Figures 3B and 3C). Especially, in the brain sections of rats receiving DSPE-mPEG2000-DiI-LNCs, the fluorescent spots were largely enhanced throughout the tissues associated with obvious fluorescence of capillary lumen. The measurements of the fluorescent surface in pixels of inclusive threshold images allowed to semi-quantify the presence of LNCs in the brain slides. As presented in Figure 4, in comparison with the control, the fluorescent surface increased significantly with DiI-LNC administration and achieved the highest value in the brain slide for rats receiving DSPE-mPEG2000-DiI-LNCs. Indeed, the fluorescent surface measured from these slides was about 1.75-fold higher than that of DiI-LNCs and about 3-fold than that of control.

By labelling cell nuclei with DAPI, the tumour was recognised by blue fluorescence (Figure 3D) whereas of DiI-labelled nanoparticles was detected by red fluorescence (Figure 3E). The merged image showed the accumulation of nanoparticles surrounding the tumour zone (Figure 3F). Consequently, these promising results could potentially lead to an improvement in the therapeutic efficacy of anticancer drug-loaded LNCs on tumour-bearing rats.

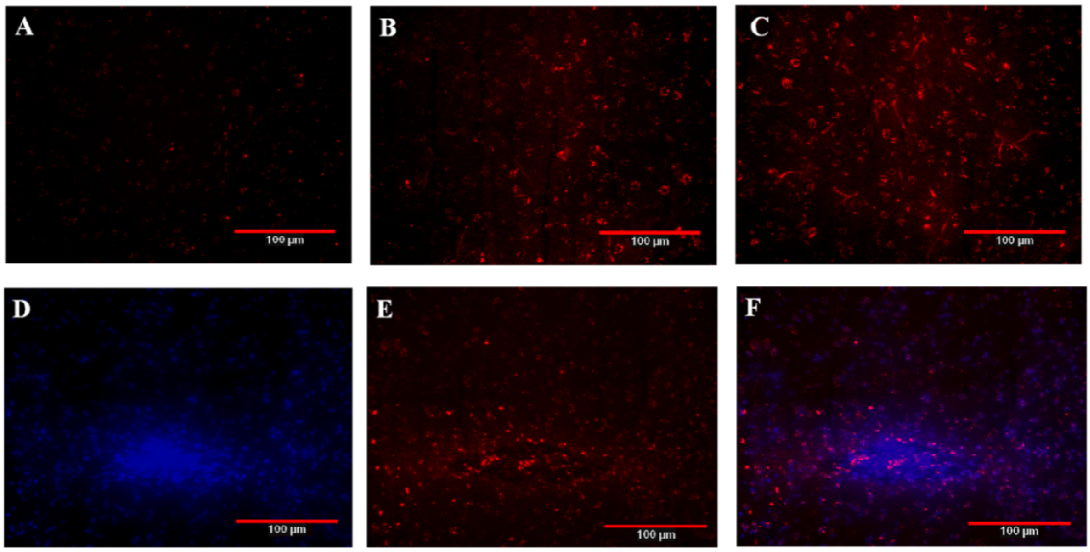


Figure 3: Section representative image under fluorescent microscope taken from the control tumour-bearing rat (A) and rats receiving an intra-carotid administration of DiI-LNCs (B) or DSPE-mPEG2000-DiI-LNCs (C). The blue-fluorescent DAPI (D) denoted the tumour zone whereas the red fluorescence of DiI (E) showed the distribution of LNCs around the tumour illustrated by the merged image (F).

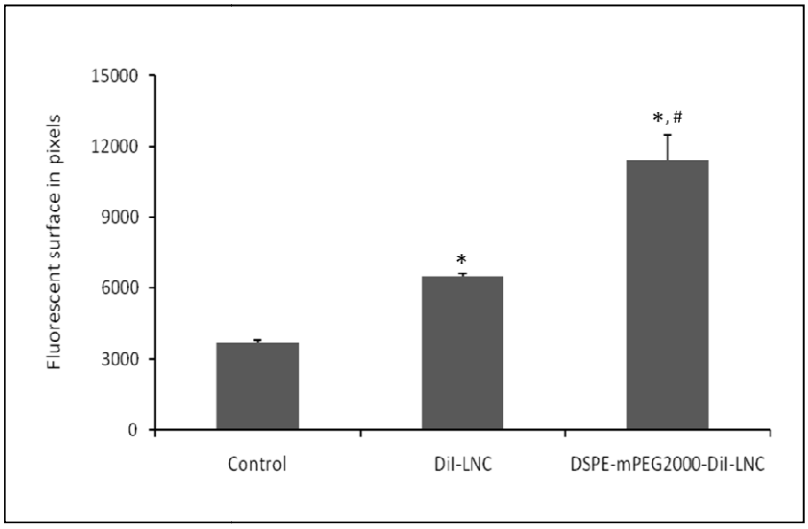


Figure 4: Semi-quantified presence of LNCs 24h after administration in the brain slides by measurement of DiI-fluorescent surfaces. (*) indicates the statistically-significant difference in fluorescent surface upon DiI-LNC and DSPE-mPEG2000-DiI-LNC administration from control rats. ($^{\neq}$) indicates the statistically-significant difference between DSPE-mPEG2000-DiI-LNC administration and DiI-LNC one.

3.3.2. Efficacy of intra-carotid treatment

Survival times of experimental animals were plotted on the Kaplan Meier curve as shown in Figure 5. Results showed that intra-carotid treatment with FcdiOH-LNCs slightly increased the survival time of treated animals (median = 27 days) and was statistically significant compared to the untreated control group (P = 0.0177), and was comparable to treatment by CED injection (P = 0.3172) (Figure 5). Treatment with DSPE-mPEG2000-FcdiOH-LNCs increased the median survival time of rats to 30 days with an IST percentage of 20% (P = 0.0008). This result was in agreement with the previously-obtained results about the enhanced fluorescent intensity measured from the images of coronal brain slides of rats receiving an intra-carotid injection of DSPE-mPEG2000-DiI-LNCs. This survival time gain is significant and indicates a real treatment benefit as, in clinical trials on human (EORTC/NCIC-protocol), the combination of Temozolomide with focal radiotherapy presents

also a 20% IST in comparison to radiotherapy alone (from 12.1 to 14.6 months) (Stupp *et al.,* 2005).

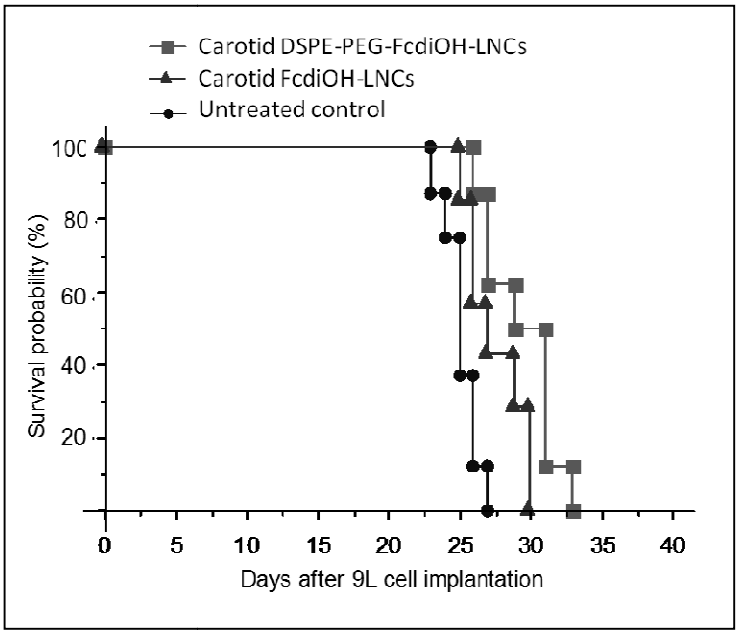


Figure 5: Kaplan–Meier survival plots for 9L glioma-bearing rats receiving intra-carotid injection of treatment at Day 6 after cell implantation. Survival times were plotted for untreated animals and treated animals with FcdiOH-LNCs or DSPE-mPEG2000-FcdiOH-LNCs (400µL of LNC suspension, corresponding to 2.4mg of FcdiOH per rat).

As a well-known characteristic, the coating with long chains of PEG renders nanoparticles 'invisible' to the immune system after injection in the blood that confers stealth properties to PEGylated nanoparticles (Huynh *et al.*, 2010). Thus, PEGylated nanoparticles have more chance to reach tumour tissues and exhibit the intrinsic activity of the entrapped drug. Previously, Morille *et al.* demonstrated the long-circulating properties of DSPE-mPEG2000-LNCs owing to their weak complement activation and low macrophage uptake associated to an extended half-life in mice as compared to conventional LNCs (Morille *et al.*, 2010).

In addition, for this administration route, crude LNC suspensions were used without the adjustment that was necessary for CED injection. Toxicity was not evaluated in this case as all the components were scattered in the blood flow, which is a different situation from what happens in CED injection. The main purpose for non adjusting LNCs was to take advantage of the hyperosmolar properties of these suspensions. Indeed, the administration of hypertonic substrates can lead to the opening of tight junctions of the BBB due to the shrinkage of endothelial cells, by which extracellular proteins are disorganised (Alam *et al.*, 2010). Consequently, drug entry takes place in a paracellular fashion and during this state, both diffusion and convection flow across the BBB can be considerably enhanced (Rapoport, 2000). Thus, nanoparticles could potentially reach the brain tumour tissues. The mechanism of brain entry as well as the reversibility and time of junction opening would be essential to determine and will be studied in further experiments.

The remaining matter will be to improve the internalisation of drug into the tumour cells after extravasation. To this end, further optimisation of the LNC surface by attaching active ligands which can specifically recognise tumour cells should be taken into consideration. For example, the covalent coupling of OX26 monoclonal antibodies to LNCs at the end of the PEG molecules leads to the formation of immunonanocapsules which specifically associate *in vitro* to cells over-expressing the transferrin receptor (Beduneau *et al.*, 2008). Indeed, OX26 targets the transferrin receptors which are highly expressed on the cerebral endothelium and also over-expressed on the surface of proliferating cells such as glioma cells (Hall, 1991). This should facilitate the passage of targeting LNCs through the BBB, followed by their internalisation into the tumour cells.

4. Conclusion

To our knowledge, this is the first report involving a neuronal, side-effect study after CED administration of a hyperosmotic infusate. At the same time, intra-arterial injection through the carotid artery was shown to represent a promising route of administration for drug delivery to the brain tumour by giving nanoparticles more chance to cross the BBB. Results revealed the importance of the physicochemical properties of the infusate versus the brain environment. In each adapted case, the benefit of regional delivery of a drug for brain cancer chemotherapy was evidenced and also confirmed the antitumour efficacy of FcdiOH in an orthotopic 9L gliosarcoma model in rats.

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PARTIE 2

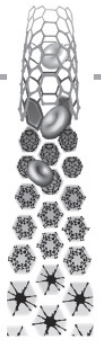
PARTIE 2

Optimisation des propriétés thérapeutiques des LNC-FcdiOH par ciblage passif ou actif

Quelque soit la voie d'injection empruntée, locale ou systémique, un vecteur de médicament administré *in vivo* va rencontrer différentes barrières physiologiques. La première barrière est son élimination rapide de la circulation systémique suite à la capture non-spécifique par le système des phagocytes mononucléés. La difficulté suivante est l'extravasation du vecteur à travers les vaisseaux sanguins. Une fois les tissus tumoraux atteints, subsistent encore la barrière cellulaire (trancytose, endocytose,…) puis la libération intracellulaire des agents thérapeutiques. Par conséquent, différentes stratégies ont été développées, par ciblage passif ou ciblage actif, afin d'aider le vecteur à franchir ces différents étapes.

Cette partie est constituée d'une revue bibliographique (revue bibliographique n°2) décrivant les avantages ainsi que les limites des nanoparticules pégylées dans le traitement systémique du cancer, par le biais d'une comparaison entre ciblage passif et ciblage actif. Ces approches sont ensuite abordées dans les travaux expérimentaux afin d'améliorer l'efficacité des LNC-FcdiOH dans le cas d'un ciblage passif (publication de résultats n°3), puis de manière préliminaire dans le cas d'un ciblage actif (publication de résultats n°4).

Revue bibliographique n°2
publiée dans Nanomedicine (Lond)
Vol 5(9), 1415-1433 (2010)



REVIEW

The rise and rise of stealth nanocarriers for cancer therapy: passive versus active targeting

Research in designing and engineering long-circulating nanoparticles, so-called 'stealth' nanoparticles, has been attracting increasing interest as a new platform for targeted drug delivery, especially in chemotherapy. In particular, the modification of nanoparticulate surfaces with poly(ethylene glycol) derivatives has illustrated a decreased uptake of nanoparticles by mononuclear phagocyte system cells and, hence, an increased circulation time, allowing passive accumulation in the tumor. The clinical trials on patients with solid tumors are described in this article, to illustrate this generation of promising nanoparticles. In the last few years, the new-generation technique of grafting ligands on the nanoparticle surface in order to target and penetrate specific cancer cells has been developed. This article discusses the benefits of passive targeting for drug delivery to the solid tumors via the enhanced permeability and retention effect, when using stealth nanoparticles, and compares them with the advantages of active targeting.

KEYWORDS: anticancer drug ■ enhanced permeability and retention effect ■ EPR effect ■ ligand ■ long-circulating nanocarriers ■ PEGylation ■ targeted nanoparticles

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Over the past few decades, interest in designing and developing nanosized drug delivery systems (also known as nanocarriers) has undergone considerable explosion. Indeed, these nanocarriers provide potential solutions to improve cancer chemotherapy by over-riding the poor biopharmaceutical properties of drugs, and by altering the pharmacokinetic and biodistribution of conventional cytotoxic molecules [1]. However, the pharmaceutical application of these systems in systemic administration is usually limited, owing to their rapid elimination from the blood circulation, resulting from a nonspecific uptake by the mononuclear phagocyte system (MPS) [2,3]. Consequently, in order to overcome the opsonization of nanocarriers, a number of widely used and effective methods have been investigated to render nanocarriers 'invisible' to the immune system, creating long-circulating nanoparticles (NPs), known as stealth NPs. Interestingly, by coating the nanoparticulate surface with poly(ethylene glycol) (PEG), referred to as a PEGylation process, NPs exhibited decreased levels of uptake by the MPS and, consequently, an increased circulation time in the blood, allowing passive targeting of the tumors [2,4]. Furthermore, surface modification of NPs with PEG moieties has emerged as a platform for the incorporation of active-targeting ligands, thereby providing the drug carriers with specific tumor-targeting properties [5].

In this article, the interest of stealth NPs will be described. Nanocarriers that will be considered include liposomes (vesicles in which an aqueous volume is entirely surrounded by a bilayer phospholipid membrane), micelles (self-assembly of amphiphiles that form supramolecular core–shell structures in the aqueous environment), polymer NPs (including nanospheres and nanocapsules) and lipid NPs (a biocompatible lipid core that is present under solid matrix [i.e., solid lipid NPs], or a liquid oily core surrounded by surfactant [i.e., lipid nanocapsules]) (FIGURE 1).

Specifically, this article classifies the various anticancer drug families incorporated into stealth NPs that have reached clinical trials. Proteins, DNA or siRNA, which may also be incorporated into PEG-coated NPs [6–8], indicating the potential application of such nanocarriers in immune and gene therapies via systemic injection, will not be described herein.

Finally, the steps from passive to active targeting will be discussed, with respect to their own advantages, as well as limitations, in anticancer drug delivery strategies. Further perspectives from the platform of stealth NPs will be discussed in the conclusion.

Passive targeting in anticancer drug delivery

■ Stealth NP paradigm

A single cancerous cell surrounded by healthy tissue will replicate itself at a rate higher than the other cells, placing a strain on the nutrient supply and the elimination of metabolic waste products. Tumor cells will displace healthy cells until the tumor size exceeds 2 mm in diameter [9]. For

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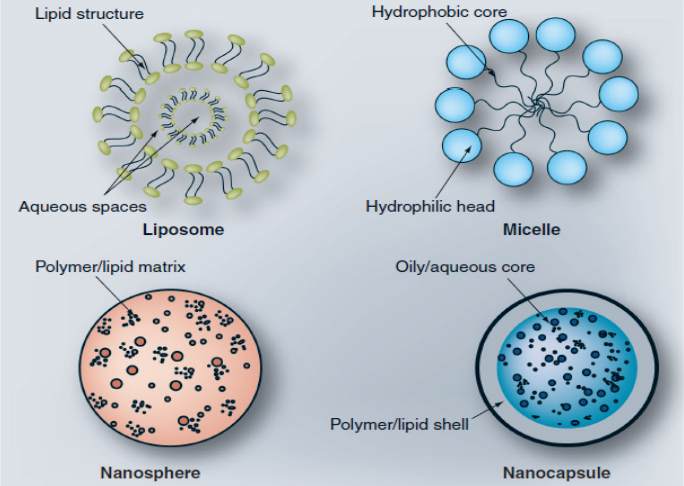

Figure 1. Different types of nanocarrier structures.

further growth and enlargement, tumors need to form new blood vessels, via the angiogenesis process, in order to obtain nutrients and sustain their growth [4]. Tumor vessels are generally abnormal, and have aberrant branching, blind loops of twisted shape, which are characterized by architectural defectiveness, and a high degree of vascular density [10]. When compared with normal vessels, tumor vessels are 'leaky', owing to basement membrane abnormalities and a decreased number of pericytes lining the rapidly proliferating endothelial cells [11]. This results in an enhanced permeability for molecule passage through the microvascular wall into the interstitium surrounding tumor cells [12]. Indeed, the pore size of tumor vessels varies from 100 nm to almost 1 µm in diameter, depending upon the anatomic location of the tumors (e.g., smaller in cranial tumors compared with subcutaneous tumors) and the tumor growth (e.g., smaller in regressing tumors) [13]. In comparison, the tight endothelial junctions of normal vessels are typically of 5–10 nm in size [13].

Moreover, solid tumors are also characterized by an impaired lymphatic network [4]. Lymphatic vessels are widely distributed throughout the body, whose major function is to return the interstitial fluid to the blood circulation. Normally, macromolecules are cleared from tissues via the lymphatic system. However, owing to the lack of lymphatic drainage, the clearance of macromolecules from solid tumors is eventually decreased and, consequently, they can have extended retention times in the tumor interstitium [14]. This, together with leaky tumor blood vessels, results in the enhanced permeability and retention (EPR) effect [4]. Therefore, passive cancer targeting is obtained through the accumulation of long-circulating nanocarriers, at a higher concentration in the tumors than in the plasma or in other tissues (FIGURE 2).

To achieve selective tumor accumulation via the EPR effect, NPs must reside for long enough in the bloodstream to provide a sufficient level of target accumulation. Unfortunately, following intravenous administration, conventional nanocarriers (first generation) have short circulation half-lives, resulting from their opsonization and interaction with the MPS. The opsonization process consists of the adsorption of protein entities, which interact with specific plasma membrane receptors on monocytes and various subsets of tissue macrophages, thus promoting particle recognition and elimination from the systemic circulation by MPS cells, mainly Kupffer cells of the liver and macrophages of the spleen [2]. This premature elimination prevents first-generation nanocarriers from reaching tumor sites by the EPR effect. Consequently, particulate surfaces must be modified for the purpose of evading the opsonization process, and the uptake by MPS cells [15]. Thus, second generation of long-circulating nanocarriers is based on the physicochemical concept of steric repulsion by grafting PEG residues or polysaccharides onto the nanoparticulate surface (TABLE 1) [16–19]. Indeed, the presence of such macromolecules allows both a steric stabilization and the prevention of opsonization thanks to a protective hydrophilic and flexible layer, which prevents aggregation between particles themselves, as well as their interaction with blood components [20]. As results, this modification of the surface of NPs generally causes a decrease in the rate of uptake by the MPS, and prolongs the biological half-life of NPs. Thus, PEGylated NPs can circulate for a longer time, and normally exhibit a circulation half-life of 2–24 h in mice and rats, and as long as 45 h in humans [2].

There are many factors influencing the opsonization and the stealth properties of these carriers, including nanoparticulate physicochemical parameters (e.g., nature of the components, size, apparent electrical charge and hydrophilicity), and characteristics of the coating itself (e.g., molecular weight, density, flexibility, conformation and copolymer composition). These different parameters in detail have already been

discussed by Vonarbourg *et al.* [16]. Briefly, preferentially NPs should be small (<200 nm), composed of biocompatible compounds and present a neutral and hydrophilic surface, with a dense and flexible coating of a polymer or polysaccharide [16]. As shown in TABLE 1, the technology of PEGylation has been successfully applied to various types of NPs. The pioneering publications on PEG liposomes were published at the end of the 1980s by Allen *et al.* [21] and the cell-specific targeting of liposomes even before, in 1980 [22,23].

In this article, our discussions will be limited to the NPs whose surfaces have been modified with PEG, the most widely used material for surface modification because it is nontoxic, nonimmunogenic and approved by the US FDA for oral and parenteral applications in humans.

Anticancer drugs loaded in stealth NPs: from animal studies to clinical trials in humans

According to the properties of anticancer drugs, the sites of interest, and the scientific and economic constraints (e.g., patents, cost of materials and final products, and scaling-up of the production processes), appropriate NPs have to be optimized. Many *in vivo* studies of stealth NPs for cancer therapy have been performed in this way, and are summarized in TABLE 2. Among them, several formulations of drug-loaded stealth NPs were approved by the FDA for clinical studies, but few of them are currently on the market (TABLE 3). The following chapter focuses on some of the clinical studies using PEGylated NPs loaded with various anticancer agents, classified by function of their drug family. Moreover, passive and active targeting strategies were grouped in TABLES 2 & 3 in order to facilitate their comparison.

Taxanes

One of the most successful cancer drugs, paclitaxel (PTX), has shown its potency against a broad spectrum of cancers, including breast, ovarian, and small-cell and non-small-cell lung cancers [24]. Another used taxane anticancer agent is docetaxel (DTX). Owing to their high hydrophobicity (aqueous solubility is 0.7 and 6–7 µg/ml for PTX and DTX, respectively), special formulations were prepared for intravenous administration. PTX formulations (e.g., Taxol® and Paxen®) consist of a mixture of absolute alcohol and Cremophor EL®; DTX (Taxotere®) is present as a concentrated viscous solution in polysorbate 80 (Tween® 80). However, toxicological side effects of these current commercial formulations of taxanes were observed, owing to the presence of these excipients [25,26]. Consequently, the clinical use of both formulations is recommended, in association with premedication with corticosteroids and H1/H2 antihistamines (for PTX) to minimize the side effects [24].

Therefore, many strategies to design alternative formulations, for both PTX and DTX, have been under investigation. Among potential approaches to achieve a desired formulation by various methods (e.g., cosolvency, emulsification, micellization, local drug delivery devices [e.g., pastes and implants]) [27], nanotechnology offers a promising solution for chemotherapy with taxanes. Recently, Abraxane®, an albumin-bound NP formulation of PTX, was granted FDA approval in January 2005. Such drug-conjugated albumin formulations are also considered as an attractive approach for selective drug targeting by improving the pharmacokinetic profile of anticancer agents, owing to the long

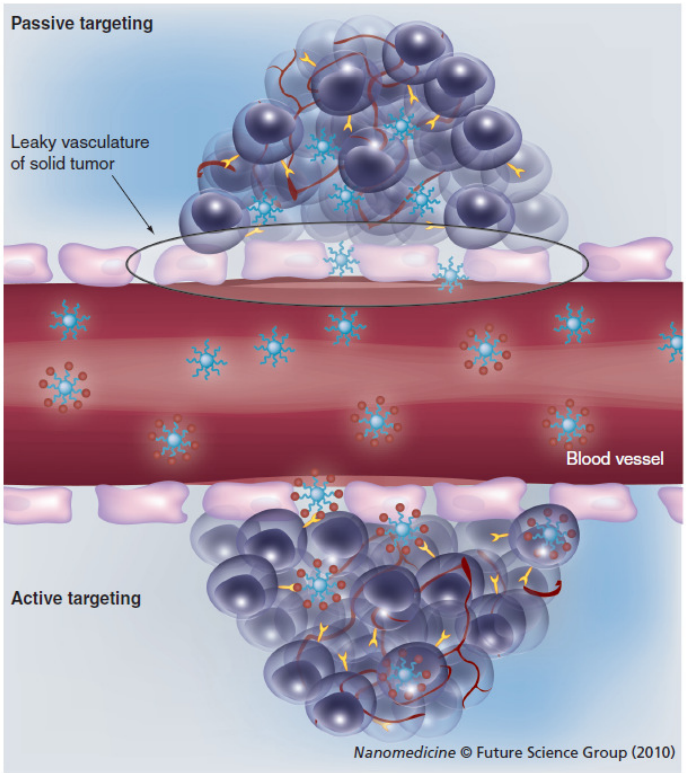

Figure 2. Passive targeting and active targeting for drug delivery to solid tumors.

Table 1. Various coatings used for the preparation of stealth nanoparticles.

Group	Coating	Type of NP	Ref.
PEG	PEG		[137]
PEG–lipid conjugates	DSPE–PEG	Liposomes LNCs	[51,60,61,138–141] [6,142,143]
	DPPE–PEG	Liposomes SLNs	[144] [145]
	Acid stearic PEG	SLNs LNCs	[91,145–147] [148]
Block copolymers	PEG–PLA	Polymer NPs Micelles	[149–153] [30,154]
	PEG–PCL (poly-ε-caprolactone)	Polymer NPs	[155]
	PEG–PLGA	Polymer NPs	[155–162]
	PEG–poly(hexadecylcyanoacrylate)	Polymer NPs	[163–168]
	PCL–PEG–PCL	Polymer NPs	[169–171]
	PLA–PEG–PLA	Polymer NPs	[172–174]
	PEG–PLA–PEG	Polymer NPs	[78,152,174]
	Poloxamer®/Poloxamine®	Polymer micelles Polymer NPs	[44] [150,151,175]
Other polymers	Poly(amino acid)		[88,176]
	Oxazoline-derived polymers		[177]
	Polyvinyl pyrrolidone		[178,179]
Polysaccharides	Chitosan		[77,180,181]
	Dextran		[72,182,183]
	Heparin		[72,181,183]

DPPE: Dipalmitoyl-phosphatidylethanol-amine; DSPE: Distearoylphosphatidylethanolamine; LNC: Lipid nanocapsules; NP: Nanoparticle; PCL: Polycaprolactone; PEG: Polyethylene glycol; PLA: Polylactic acid; SLN: Solid lipid nanoparticles.

half-life of albumin in the body [28,29]. However, we do not discuss this in detail because their surfaces were not coated with PEG to confer the stealth properties.

Another example of a Cremophor-free formulation of PTX is Genexol®-PM, a polymer micellar system prepared with methoxy-PEG–poly(lactide) (mPEG–PLA)-block copolymers. *In vivo* studies on mice, following intravenous injection, demonstrated considerable accumulation of Genexol-PM in subcutaneously implanted B16 melanoma tumor, and a significant antitumor effect against SKOV-3 human ovarian cancer and MX-1 human breast cancer when compared with Taxol [30]. In a Phase I study, in which patients received Genexol-PM intravenously without premedication for 3 h once every 3 weeks, the maximum tolerated dosage (MTD) of 390 mg/m^2 was obtained [31]. The main dose-limiting toxicities of Genexol-PM were neuropathy, myalgia and neutropenia, with an absence of acute hypersensitivity reactions. The Phase II trials showed that Genexol-PM was tolerated and effective in patients with advanced pancreatic cancer [32], metastatic breast cancer [33], and also in patients with advanced non-small-cell cancer who were administered a combination of Genexol-PM and cisplatin [34]. Genexol-PM is now approved in South Korea [35], and is undergoing Phase III and IV trials in patients with recurrent or metastatic breast cancer [301].

Anthracyclines: doxorubicin/daunorubicin

Anthracyclines, including doxorubicin (DOX) and daunorubicin, are among the most active agents available for the treatment of breast cancer, and are a key component of adjuvant therapy regimens. However, the clinical use of these agents is always limited, particularly in patients receiving adjuvant treatment with anthracyclines during the early phases of breast cancer, owing to their cumulative dose-related cardiotoxicity [36]. Therefore, it is necessary to exploit drug delivery systems to confer altered biodistribution and drug-release properties to minimize cardiotoxicity and maximize drug delivery to tumor tissues.

One striking success of DOX-loaded stealth NPs is the case of Doxil® (in the USA) or Caelyx® (in Europe) in 1995, which was the first nanocarrier approved by the US FDA and the European Medicines Agency (EMA) for cancer therapeutics. This is a DOX-containing formulation based on stealth liposomes, which,

Table 2. Preclinical studies using stealth nanoparticles in cancer chemotherapy.

Stealth NPs	Targeting type	Animal	Findings	Ref.
Paclitaxel				
DSPE–mPEG2000-liposomes	Passive	Rat, nude mouse	$T_{1/2\beta}$ = 17.8 vs 5.05 h for uncoated liposomes, and 1.65 h for Taxol® in rats. Enhanced antitumor effect due to high accumulation in MDA-MB-231 human breast cancer bearing nude mice. High PTX payload with an efficiency level up to 70%	[139]
Anti-HER2-S_{100}PC:CHOL:mPEG2000-DSPE:Mal-PEG2000-DSPE liposomes	Active	Nude mouse	Higher ratio of tumor to plasma drug concentrations (T/P ratio) than nontargeted liposomes in HER2-overexpressing breast carcinoma model (BT-474) linked to a higher antitumor efficacy. Weak T/P ratio in low HER2-expressing model (MDA-MB-231)	[184]
DPPE-mPEG5000 liposomes	Passive	Mouse	Long circulation time ($T_{1/2\beta}$ = 48.6 h vs 9.27 h for uncoated liposomes). Considerable decrease in drug uptake in MPS-containing organs (liver and spleen) at 0.5 and 3 h after injection as compared with the conventional liposomes	[144]
mPEG–PLA NPs	Passive	Rat	Higher half-life (18.80 ± 3.14 h) and AUC than Taxol (2.75- and 3.09-times, respectively) at the same dose of 10 mg/kg. Drug encapsulation efficiency up to 87%	[153]
PLA–PEG–RGD micelles	Active	Nude mouse	Higher tumor accumulation than nontargeted micelles in a model of MDA-MB-435 breast tumor linked to a tumor regression with PTX-targeted micelles	[185]
PEG–PLGA NPs	Passive	Mouse	Remarkable tumor growth inhibition and increased survival rate of transplantable liver tumor-bearing mice compared with Taxol	[160]
Docetaxel				
PEG–PLGA NPs	Passive	Mouse	Drug biological half-life increased and tumor accumulation five-times higher than Taxotere® on C26 tumor-bearing mice	[161]
DSPE–mPEG2000 LNCs	Passive	Mouse	Long circulation time (half-life: 1.4 vs 0.3 h for Taxotere)	[143]
Doxorubicin				
Anti-HER2–DSPE-COOH-PEG2000 liposomes	Active	Rat, nude mouse	Long circulation time of immunoliposomes and PEGylated liposomes in normal rats. Increased antitumor efficacy and reduced systemic toxicity with DOX anti-HER2 immunoliposomes. Similar levels of tumor tissue accumulation in a nude mouse model of HER2-overexpressing breast cancer (BT-474) for both targeted and nontargeted liposomes	[106,107]
DSPE–PEG–maleimide–RGD liposomes	Active	Mouse	Specific interaction with tumor vasculature (intravital microscopy), not observed for nontargeted PEGylated liposomes. Inhibition of tumor growth in a doxorubicin-insensitive murine C26 colon carcinoma model by DOX RGD-immunoliposomes. No growth deceleration with DOX nontargeted liposomes	[121]
SPC:CHOL:DSPE-PEG:DSPE–PEG–RGD liposomes	Active	Nude mouse	Similar drug accumulation in tumors in a model of murine B16 melanoma between targeted and nontargeted liposomes. Higher antitumor effect for DOX immunoliposomes than nontargeted liposomes	[186,187]
Folate-targeted PEGylated liposomes	Active	Mouse	Similar tumor accumulation between targeted and nontargeted liposomes in BALB/c mice with high folate receptor-expressing tumors (mouse M109, human KB carcinomas)	[188]
mAb 2C5-modified PEGylated liposomes (mAb 2C5-modified Doxil®)	Active	Nude mouse	Significant therapeutic benefit showing substantial decrease of tumor size and doubling of survival time provided by 2C5-immunoliposomes in intracranial U-87 MG brain tumors in nude mice over control formulations	[103]
PEG–PLGA NPs	Passive	Mouse	Remaining 40% injected dose in the serum of BALB/c mice 24 h postinjection. Decrease in cardiotoxicity	[156]
PCL–PEG–PCL NPs	Passive	Mouse	Significant antitumor effect on a subcutaneous C-26 tumor	[171]
Folate–PLGA–PEG micelles	Active	Nude mouse	Higher drug concentration in tumor and higher antitumor effect than nontargeted micelles in a model of KB human squamous cell carcinoma	[189]

AUC: Area under the concentration–time curve; DOX: Doxorubicin; DPPE: Dipalmitoyl-phosphatidylethanol-amine; DSPE: Distearoylphosphatidylethanolamine; mAb: Monoclonal antibody; mPEG: Methoxy polyethylene glycol; MPS: Mononuclear phagocyte system; NP: Nanoparticle; PCL–LA–PEG–PCL–LA: Poly(caprolactone-co-lactide)-b-PEG-b-poly(caprolactone-co-lactide); PLA: Poly(lactide); PLGA: Poly(lactic-co-glycolic acid); PTX: paclitaxel; RGD: Arginine–glycine–aspartate; S_{100}PC:CHOL:mPEG2000–DSPE:Mal-PEG2000–DSPE: Soybean phosphatidylcholine:cholesterol:1,2-distearoyl-sn-glycero-3-phospho-ethanolamine [methoxy(polyethyleneglycol)-2000]:maleimide-derivatized PEG2000-DSPE.

Table 2. Preclinical studies using stealth nanoparticles in cancer chemotherapy.

Stealth NPs	Targeting type	Animal	Findings	Ref.
Doxorubicin (cont.)				
Stearic acid-PEG2000 SLNs	Passive	Rat, rabbit	Higher half-life time and plasma AUC, decrease of the DOX heart concentration compared with free-drug commercial solution. Highest concentration of DOX in the blood obtained with the highest concentration of stealth agent	[91,146]
Glycol chitosan NPs	Passive	Mouse	Amount of NPs gradually increased in tumor as blood circulation time increased. Concentration in blood 14% of dose at 1 day, and 8% 3 days after intravenous injection. DOX-glycol chitosan NPs exhibited lower toxicity but comparable antitumor activity to free DOX	[190]
Cisplatin				
mPEG–PLGA NPs	Passive	Mouse	Decrease of tumor volume and higher survival rate in HT-29 tumor-bearing SCID mice than free drug, and good tolerance by normal BALB/c mice	[155]
Camptothecin analogs				
10-Hydroxycamptothecin-loaded PCL–LA–PEG–PCL–LA NPs	Passive	Mouse	Superior antitumor effect and extended retention time in the blood of S-180 (murine sarcoma)-bearing mice (>22 ng/ml detected at 24 h postinjection, whereas free drug injection was undetectable)	[169,170]
Topotecan loaded anti-HER2 and anti-EGF receptor liposomes	Active	Mouse	Improved antitumor activity against HER2-overexpressing human breast cancer (BT474) xenografts	[191]

AUC: Area under the concentration–time curve; DOX: Doxorubicin; DPPE: Dipalmitoyl-phosphatidylethanol-amine; DSPE: Distearoylphosphatidylethanolamine; mAb: Monoclonal antibody; mPEG: Methoxy polyethylene glycol; MPS: Mononuclear phagocyte system; NP: Nanoparticle; PCL–LA–PEG–PCL–LA: Poly(caprolactone-co-lactide)-b-PEG-b-poly(caprolactone-co-lactide); PLA: Poly(lactide); PLGA: Poly(lactic-co-glycolic acid); PTX: paclitaxel; RGD: Arginine–glycine–aspartate; S_{100}PC:CHOL:mPEG2000–DSPE:Mal-PEG2000–DSPE: Soybean phosphatidylcholine:cholesterol:1,2-distearoyl-sn-glycero-3-phospho-ethanolamine [methoxy(polyethyleneglycol)-2000]:maleimide-derivatized PEG2000-DSPE.

consequently, has been approved for AIDS-related Kaposi's sarcoma, and is now commercially available for the treatment of metastatic breast cancer, advanced ovarian cancer and multiple myeloma [37,38]. Preclinical and clinical studies have shown that Doxil preferentially accumulated in tumor xenografts and human tumors, resulting in an enhancement of drug concentration in the tumor compared with free DOX [39]. The plasma area under the concentration–time curve (AUC) was at least 60-fold higher in animals, and approximately 300-fold higher in humans, than free DOX. Doxil toxicity is characterized by dominant and dose-limiting mucocutaneous toxicities (stomatitis, hand-foot syndrome or palmar-planta erythrodysesthesia [40]), mild myelosupression and minimal alopecia, but no apparent cardiac toxicity [41]. The reduced cardiotoxicity allows a larger cumulative dose than that acceptable for free DOX. Therefore, Doxil can be effectively and safely substituted for conventional DOX, allowing retreatment with an anthracycline in the metastatic setting [42].

Other nanoparticulate systems have also been investigated to encapsulate DOX. SP1049C, a micellar formulation containing DOX and two nonionic block copolymers, Pluronic® L-61 and Pluronic® F-127, can be used for the treatment of primary and relapsed tumors. First, in preclinical studies, SP1049C (micellar DOX) demonstrated higher antitumor activity than free DOX in DOX-resistant tumors and in multidrug-resistance (MDR) cells [43,44]. The authors explained this higher activity of micellar DOX, in comparison with free DOX, by mechanistic hypotheses: the micellar formulation increases the drug uptake; Pluronic L-61 (one of the two copolymers used in the formulation) inhibits P-glycoprotein efflux, which normally limits the free-drug uptake and, consequently, micellar formulation increases the drug uptake; and, finally, the micellar formulation changes the intracellular drug trafficking. Then, in the Phase I study, the pharmacokinetic profile of SP1049C showed a slower clearance than free DOX [45]. The principal dose-limiting toxicity was neutropenia, and the MTD was 70 mg/m², whereas the MTD of free DOX was 50 mg/m² [46]. Moreover, recent results from a Phase II study in patients with advanced adenocarcinoma of the esophagus and gastro–esophageal junction, demonstrated that SP1049C has a notable single-agent activity. The objective response was 47% for the evaluate patients with the micellar formulation, whereas it was 43% in the

Table 3. Examples of anticancer drugs loaded in stealth nanocarriers in clinical trials or approved for cancer therapy.

Compound	Formulation (carrier/size)	Company	Indication	Targeting strategy	Route of administration	Status	Ref.
Taxanes							
Paclitaxel	Genexol®-PM: polymer micelles mPEG–PLA; 20–50 nm	Samyang, Daejeon City, Korea	Advanced pancreatic cancer, metastatic breast cancer	Passive	Intravenous	Approved in South Korea, Phase III/IV	[31–35,301]
	Nanoxel®: polymer micelles poly(vinyl pyrrolidone)-b-poly(*N*-isopropyl acrylamide); 80–100 nm	Fresenius Kabi Oncology Ltd, Haryana, India	Advanced breast cancer	Passive	Intravenous	Approved in India Phase I	[192,193]
	NK105 polymer micelles; 85 nm	Nippon Kayaku Co.,Ltd, Tokyo, Japan	Colon and gastric cancer	Passive	Intravenous infusion	Phase I	[194]
Docetaxel	Docetaxel-polymer nanoparticles; 20–50 nm	Samyang, Daejeon City, Korea	Advanced solid malignancies	Passive	Intravenous	Phase I	[195,302]
Anthracyclines and analogs							
Doxorubicin	Doxil®/Caelyx®: PEGylated liposomes; 80–90 nm	Ortho Biotech, PA, USA; Schering-Plough, QC, Canada	Kaposi's sarcoma, refractory breast cancer and refractory ovarian cancer	Passive	Intravenous/ intramuscular	Approved	[196,197]
	SP1049C: polymer micelles Pluronic® block copolymers; 22–27 nm	Supratek Pharma, QC, Canada	Esophageal carcinoma	Passive	Intravenous	Phase II/III	[45,47]
	NK911: polymer micelles from a copolymer of PEG and DOX-conjugated poly(aspartic acid); 40 nm	SupraLife International, CA, USA	Solid tumors	Passive	Intravenous infusion	Phase I	[198]
	MCC465: PEGylated antibody-liposomes; 143 nm	Mitsubishi Pharma Corporation, Osaka, Japan	Metastatic stomach and colorectal cancer	Active	Intravenous infusion	Phase I	[199,200]
Mitoxantrone (anthracenedione)	ΔpH MTO-liposomes: PEGylated liposomes; 123 nm	University Hospital of Zurich, Switzerland	Advanced breast cancer	Passive	Intravenous	Phase I	[201]
Metal-based drugs							
Cisplatin	SPI-077®: mPEG liposomes; 110 nm	Alza Corporation, CA, USA	Head and neck cancer, lung cancer	Passive	Intravenous	Phase II	[52–58,202]
	Lipoplatin®: PEGylated liposomes; 110 nm	Regulon Inc., CA, USA	Multiple cancer indications	Passive	Intravenous	Phase II/III	[203,204]
	Nanoplatin® (NC-6004): polymer micelles; 30 nm	NanoCarrier Co., Ltd, Kashiwa City, Japan	Multiple cancer indications	Passive	Intravenous infusion	Phase I/II	[205]
Oxaliplatin	MBP-426: Transferrin-liposomes; 100 nm	Mebiopharm Co., Ltd, Kanagawa, Japan	Advanced or metastatic solid tumors, colorectal, gastric and esophageal cancer	Active	Intravenous infusion	Phase II	[303]
Camptothecin analogs							
CKD602	S-CKD602: PEGylated liposome; 100 nm	Alza Corporation, CA, USA	Multiple cancer indications (advanced solid tumors)	Passive	Intravenous	Phase I/II	[62,206]
SN38	NK012: polymer micelles PEG–poly(glutamic acid) block copolymer; 20 nm	Nippon Kayaku Co.,Ltd, Tokyo, Japan	Triple-negative breast cancer and colorectal cancer	Passive	Intravenous	Phase II	[207,208]

DOX: Doxorubicin; mPEG: Methoxy-polyethylene glycol; PEG: Polyethylene glycol; PLA: Poly(lactide).

intent-to-treat population. The overall median survival and the progression-free survival were 10.0 and 6.6 months, respectively [47], while median survival of free DOX was not available. Nevertheless, with conventional therapy, which combines 5-fluorouracil with cisplatin, the median survival was 7 months, with epirubicin, cisplatin and 5-fluorouracil (ECF), the median survival was 8.9 months [48], and with 5-fluorouracil, doxorubicin and methotrexate, the median survival was 5.7 months [49].

Metal-based drugs

Cisplatin is one of the leading metal-based drugs used for the treatment of many malignancies. However, its expansion in clinical application is limited, owing to significant side effects, such as acute nephrotoxicity and chronic neurotoxicity [50]. Therefore, extensive efforts have been devoted to the specific delivery of cisplatin to tumor tissues. SPI-077 is a formulation of cisplatin, which is encapsulated in mPEG liposomes for the purpose of specific drug delivery and decreased cisplatin-related toxicity. Preclinical studies in mice demonstrated that SPI-077 exhibited increased efficacy against Lewis carcinomas and C26 colon carcinomas in BALB/c mice, and was well tolerated with high cumulative doses [51]. Unfortunately, results from different Phase I and II studies of SPI-077 demonstrated the lack of efficacy of this formulation, since no response rates, or only modest response rates, were observed in patients with non-small-cell lung cancer or advanced head and neck cancer [52–58].

Camptothecin analogs

A camptothecin analog, 7-[2-(*N*-isopropylamino)ethyl]-(20S)-camptothecin, or CKD-602, was synthesized, and proven to be more potent than topotecan in 14 out of 26 human cancer cell lines tested [59]. CKD-602-loaded stealth liposomes (S-CKD-602) demonstrated prolonged circulation time in the plasma, resulting in threefold longer drug exposure in tumors, and improved antitumor efficacy compared with free drugs in nude mice bearing human tumor xenografts [60,61]. Recently, this stealth liposomal formulation of CKD-602 has entered a Phase I study in patients with advanced malignancies [62]. It was well tolerated, with a MTD of 2.1 mg/m^2. The overall incidence of grade 3 or 4 toxicity was comparable to other camptothecins. Therefore, the Phase II studies of S-CKD-602 at a dose of 2.1 mg/m^2, administered intravenously once every 3 weeks, have been planned [62].

From passive to active targeting

Drawbacks of passive targeting

Most stealth NPs are expected to accumulate in the tumor site owing to the passive targeting. This passive targeting is, hence, highly dependent on the degree of tumor vascularization and angiogenesis [63,64]. Unfortunately, certain tumors do not exhibit the EPR effect. Moreover, the permeability of vessels throughout a tumor, and between different tumor types, may not be the same [65]. Indeed, the porosity and pore cut-off size of tumor vessels varies with tumor type (range: 0.3–4.7 mm), host microenvironment (cranial window or dorsal chamber), vascular structure and degree of tumor maturity [66–68]. The same tumor, grown subcutaneously or orthotopically, can show very different EPR effects [69]. Consequently, considerable knowledge of the nature of tumor vascularization and tumor type is required to administer anticancer treatment at the right time. Moreover, many solid tumors possess a high level of interstitial fluid pressure, which limits the transport of nanocarriers and/or drug through the interstitial spaces upon extravazation and, therefore, inhibits efficient uptake and homogenous distribution of nanocarriers and/or drug in the tumor tissues [70]. Different factors that affect EPR effects are well reviewed by Maeda *et al.* [64], and some alternatives have been proposed to increase the EPR effect and drug delivery to the tumor (i.e., angiotensin II coadministration) [68]. If experimental animal tumor models, characterized by increased vascular permeability, are commonly used in the literature, the situation, most likely, is very different in cancer patients, and only clinical results will provide the right answer.

In parallel, polymers used to confer stealth characteristics to NPs present some limitations. An important consideration in clinical therapeutics is the ultimate fate of polymer constituents used in NP engineering [2]. Little work has been carried out in determining the clearance mechanism, the metabolism and excretion of NPs, and their components [20], even when the chemical characterization of polymers was studied [71]. Therefore, more studies are required to elucidate this matter. Indeed, PEG inhibits MPS uptake, but does not block this process, contrary to certain polysaccharides, such as heparins, which are known for their complement-inhibition properties [72]. Consequently, some NPs and/or free polymer chains of PEG could be taken by the MPS, and could be responsible for possible toxicity, since, despite the fact that low-molecular-weight PEG has been shown to

be nontoxic, and readily excreted by the kidneys, it is a nonbiodegradable polymer. It cannot be excluded that, after cellular uptake, PEG affects cell functioning at the long-term follow-up after internalization in the macrophages [73–75], as well as in liver, spleen, kidney and lung cells, where a considerable fraction of NPs can be found [76–78]. For example, some polymers may elicit immune response or allergic reactions, which are more likely following subsequent injections, although they may be controlled by corticosteroids and antihistamines. There were previous reports on the so-called 'macromolecular syndrome' or, more lately, on the infusion-related reaction, which is observed at first usage, even with antibody drugs or liposomal drugs, and is not observed in the second administration [64].

On the other hand, Ishida *et al.* reported that PEGylated liposomes lost their long-circulating property when they were administered twice in the same animal, and accumulated extensively in the liver [79]. This is referred to as the 'accelerated blood clearance' (ABC), the mechanism of which is related to the abundant production of anti-PEG IgM from the spleen in response to the first injection of PEGylated liposomes [80]. The IgM can selectively bind to the PEG chains on a second injection, administered several days later, and subsequently activates the complement system and enhances uptake of the second PEGylated liposome injection by the Kupffer cells [81,82]. Consequently, this ABC phenomenon may decrease the therapeutic effect of the loaded drug, and potentiate the adverse effects or even induce the morbidity [83,84]. This was also reported with other PEGylated nanocarriers, for example PEG–PLA NPs [85,86] and polymer micelles [87]. Therefore, further evidence is required to elucidate whether the ABC mechanism is induced by all kinds of PEGylated NPs, and if it can occur in humans. Recently, it was observed that the ABC phenomenon could be prevented by changing the properties of the PEG lipid derivatives or by replacing the coating by other polymers (i.e., poly[amino acid]) [88]. Judge *et al.* [89] and Semple *et al.* [83] demonstrated the lack of this phenomenon by using PEG-conjugated ceramides (PEG–Cer), with a small C14 lipid anchor in modified liposomes. Xu *et al.* demonstrated that the use of other cleavable PEG–lipid derivatives could also prevent ABC [90]. Indeed, repeated injection of conventional distearoylphosphatidylethanolamine (DSPE) PEG liposomes resulted in the enhanced elimination of liposomes, accompanied by increased accumulation in the liver. By contrast, the pharmacokinetic and biodistribution profiles after two injections of cholesteryl hemisuccinate (CHEMS) PEG liposomes were comparable to a single injection.

Finally, modifying the physicochemical properties of the NP surface, in order to render them as stealth NPs, in parallel can bring important drawbacks. For example, increasing the concentration of PEG is known to influence the surface polymer conformation and reduce the zeta potential of the nanocarriers in an interesting manner, but also increases their average diameter [6,91], which can negatively influence the biodistribution of NPs [16]. More specifically, it can also limit cellular uptake by the endocytotic route [92]. As mentioned, a high density of PEG on the NP surface is necessary to improve *in vivo* blood circulation of the NPs, but the abundant presence of PEG may hinder the binding and uptake of nanocarriers by cancer cells by preventing nanocarrier/tumor cell contact [93]. Indeed, although PEGylated NPs frequently show superior *in vivo* antitumor activity, they are generally less cytotoxic *in vitro* than conventional NPs [94,95], owing to lack of interactions. Moreover, since most cytotoxic anticancer agents act intracellularly, the release of entrapped drugs from NPs must occur inside the tumor cells. Rapid leakage of the drug payload in the blood circulation may lead to a decrease in drug efficacy and potentiate an undesirable effect. Once they have arrived at the target tumor site, nanocarriers can release their content in close proximity to the target cells (i.e., in the case of passive targeting), attach to the membrane of the cell via nonspecific receptors and act as an extracellular sustained-release drug depot, or internalize into the cell before the drug is released owing to specific receptor-mediated internalization (FIGURE 3). When biopharmaceutical agents are not membrane permeable, such as proteins, peptides or nucleic acids, the extracellular release of the drug is not expected, and it is required that carriers are internalized to the target tumor cell in order to deliver the encapsulated drug to the appropriate location inside the cell. Therefore, the enhanced intracellular delivery by using PEGylated NPs without loss of drug activity (i.e., the improved bioavailability of drug), is a key step in drug delivery, which still remains a scientific challenge. As a consequence, in order to increase tumor localization, as well as tumor cell internalization, third-generation nanocarriers based on specific targeting have been developed.

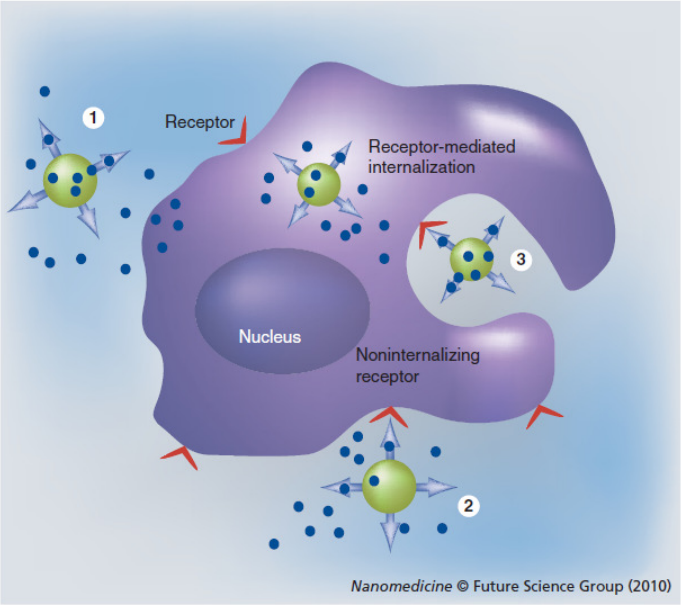

Figure 3. Mechanism of the drug release when reaching the tumor site (1–3).

Active ligand-conjugated stealth NPs

Active targeting involves the use of peripherally conjugated targeting moiety (or ligand) for enhanced selective cellular binding due to specific interactions with receptors overexpressed on tumor cells. In some cases, it can be followed by the internalization of NPs through receptor-mediated endocytosis [96]. The targeting moieties are generally attached onto the surface of the colloidal particles, preferably at the end of the PEG molecules, since the targeted colloidal particles will be much more efficient if they are also sterically stabilized and, of course, considered stealth NPs [97]. Various targeting moieties have been employed for drug delivery systems, for example antibody fragments, peptides, sequences identified by phage display, small molecules or aptamers (FIGURE 4) [98,99]. Detailed descriptions of some active targeted NPs are depicted to illustrate the description of the ligands below.

Antibodies

Monoclonal antibodies (mAbs) or whole mAb fragments, including Fab' and single-chain Fv (scFv) fragments, have been widely used as ligands to target receptor-expressing cancer cells [100]. For example, an *in vitro* study by Kocbek *et al.* demonstrated that poly(lactic-co-glycolic acid) (PLGA) NPs conjugated with mAbs were localized directly into MCF-10A neoT cells, whereas noncoated NPs were distributed randomly [101]. Elsewhere, mAb 2C5-modified PEGylated liposomes, demonstrated three- to eight-fold increase in binding and internalization by various cancer cell lines of diverse origins, higher cytotoxicity towards various cancer cells and a significant therapeutic benefit over control (liposomes modified with a nonspecific IgG) [102,103]. Nevertheless, antibody fragments Fab' and scFv, rather than intact mAbs, were frequently used to minimize immunogenicity, and to circumvent clearance via Fc receptor-mediated mechanisms [98,104]. Kou *et al.* developed PLGA NPs coated with SM5–1 monoclonal antibody (scFv), which enhanced *in vitro* cytotoxicity against human hepatocellular carcinoma cell lines, and significantly inhibited tumor development and induced tumor regression [105]. Herceptin® is another therapeutic antibody targeting human EGF receptor-2 (HER2), which is overexpressed on breast cancer cell surfaces [100]. Studies using anti-HER2 immunoliposomes, by conjugating anti-HER2 antibody fragments to PEGylated liposomes, demonstrated that anti-HER2 immunoliposomes achieved intracellular drug delivery via mAb-mediated endocytosis, whereas nontargeted liposomes were predominantly found in extracellular stroma or within macrophages [106]. DOX-loaded anti-HER2 immunoliposomes also showed significant antitumor effect compared with nontargeted liposomes [107]. However, although the authors demonstrated a high uptake of these immunoliposomes, the role of the active targeting is questionable. Indeed, in the same paper [108], the authors showed similar high levels of tumor tissue accumulation in HER2-overexpressing breast cancer xenografts (BT-474) between anti-HER2 immunoliposomes and nontargeted liposomes. Thus, in this case, targeted nanocarriers did not augment the tumor accumulation, as compared with nontargeted ones. The first explanation on the lack of accumulation is the type of model used in this paper. The study was performed with tumor cells, and not with tumor endothelial cells, which are the target. Another explanation is the high density of ligands on the NP surface. Indeed, the presence at a high density of active ligands can prevent the long-circulating properties of PEG, and lead to an accelerated elimination of NPs from the blood circulation [108]. Moreover, in order to use antibodies as a targeting moiety, different factors should be taken into consideration [96,109–112]:

- The method of conjugation to incorporate antibodies to nanocarriers (i.e., conjugation could modify the activity of the antibody and its receptor affinity);
- The consequence of circulating free antibody;
- The immunogenicity of these entities, since most of them are derived from animals and are easily recognized as foreign, which can cause strong immune responses;
- The nonspecific binding of antibodies via Fc fragments, and the possible changes in the antigen over time.

For example, Matsku *et al.* demonstrated that immunoliposomes have limited accumulation in solid tumor tissue owing to their moderate stability (the targeted mAb was released from liposomes within a few hours) and their inability to extravasate [113]. Thus, these different disadvantages of using antibodies as ligand moieties could explain the lack of commercial antibody-conjugated NPs applied in therapy, even if most antibodies have been approved by the FDA and/or the EMA [114].

Nucleic acid aptamers

Besides antibodies, nucleic acid aptamers, which are single-stranded oligonucleotides, either RNA or DNA, have also been used as targeting moieties [115]. Aptamers can be produced and modified easily, exhibit no intrinsic toxicity or immunogenicity, and are easy to handle [116]. The main disadvantages of using aptamers are the high costs to generate them, especially in large quantities, and their susceptibility to enzymatic degradation [114]. A10 RNA aptamers, which bind to the prostate-specific membrane antigen (A10 PSMA aptamers), conjugated to PEG–PLA NPs, exhibited a high capacity for *in vitro* active binding and uptake by prostate LNCaP epithelial cells, which express the PSMA protein [117]. Moreover, DTX-loaded PEG–PLGA NP–aptamer conjugates showed remarkable *in vivo* antitumor effect and reduced toxicity on a subcutaneous LNCaP xenograft mouse model after a single intratumor injection [118]. The biodistribution upon a retro-orbital injection in a similar animal model of prostate cancer demonstrated significantly enhanced delivery of NP–aptamer conjugates to the tumors with 3.77-fold higher levels than control NPs lacking the A10 PSMA aptamer [119].

Peptides

Several internalizing peptides, as well as the phage-display peptide libraries, have also been used as targeting ligands, such as integrin-binding peptides containing arginine–glycine–aspartate (RGD) domains [120]. By coupling cyclic RGD peptides to the distal end chain of PEG-coated liposomes, Schiffelers *et al.* showed that cyclic RGD peptide-modified liposomes exhibited increased *in vitro* binding to endothelial cells [121]. The encapsulation of DOX into such targeted liposomes inhibited tumor growth in a DOX-insensitive murine C26 colon carcinoma model [121]. In this case, the therapeutic effect of RGD peptide-modified liposomes was not explained by the cytotoxic effects on tumor cells, but it resulted from the inhibition of tumor progression via inhibition of angiogenesis owing to the targeted delivery of DOX to endothelial cells. More recently, Sugahara *et al.* demonstrated that NPs coated with a tumor-homing peptide, iRGD, enhanced the tumor penetration of the drug [122]. Indeed, the peptide mediated specific binding to α_v-integrins on tumor endothelium, followed by a proteolytic cleavage, which exposed a binding motif for neurophilin-1, mediating the penetration of NPs/drug into tissue and cells.

Small molecules

The use of small molecules, such as folic acid and sugar molecules, as targeting ligands seems to be an attractive approach owing to their low cost and easy conjugation with drugs [123]. All cells require folic acid as a vitamin for essential functions, and folate receptors are overexpressed

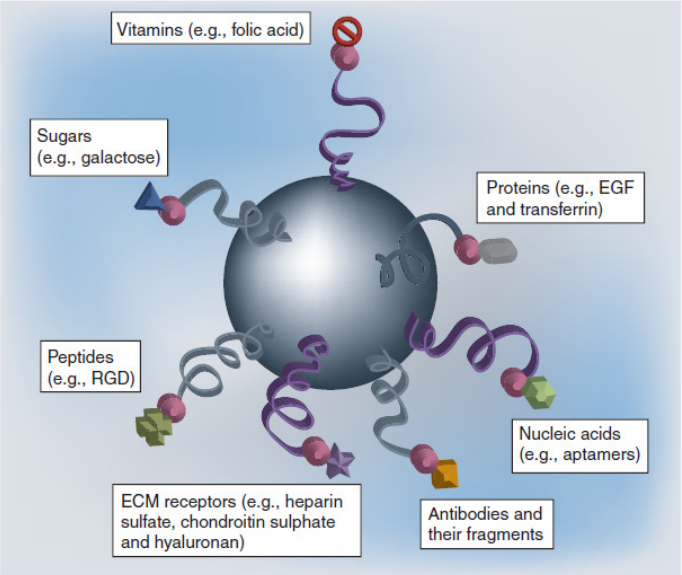

Figure 4. Types of active ligands used for active targeting.
RGD: Arginine–glycine–aspartate.

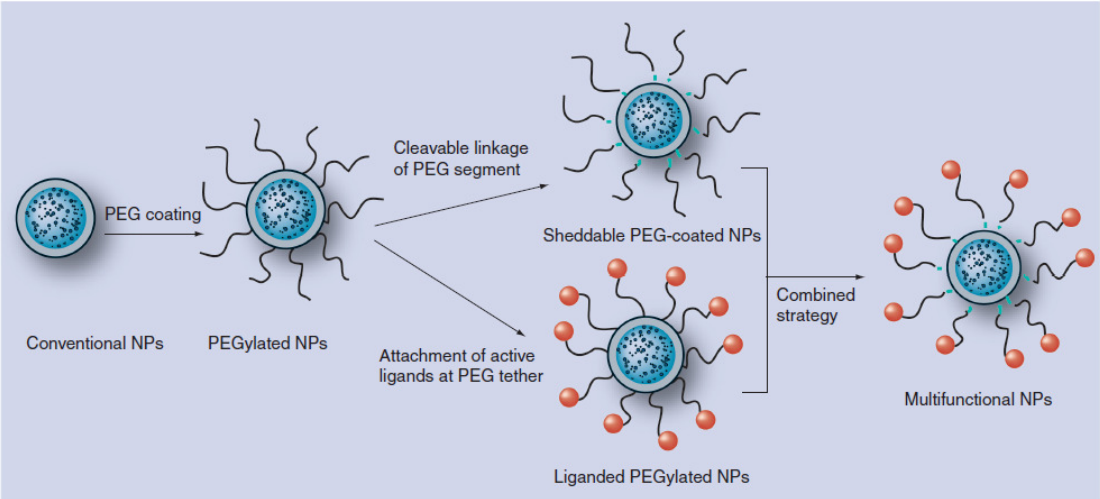

Figure 5. Different strategies for surface modifications from the conventional nanoparticles.
NP: Nanoparticle; PEG: Poly(ethylene glycol).

in many cancer cells that require folic acid for their rapid proliferation [124]. Folate-targeted liposomes have been shown to be effective carriers for intracellular delivery of nucleic acids and anticancer drugs to *in vitro* tumor cells [124]. Similarly, cell surface membrane lectins are also overexpressed at the surface of numerous cancer cells and able to specifically internalize sugar molecules via receptor-mediated endocytosis mechanisms, such as the mannose or fructose receptor of macrophages, and the asialoglycoprotein receptors of hepatocytes [125,126].

In summary, it is noteworthy that active targeting using stealth NPs functionalized with ligands that have high-affinity levels and high specificity promises to be a powerful approach, leading to effective drug delivery into tumor cells for the treatment of cancer.

Conclusion & future perspective

After intravenous administration, pharmacological activity of anticancer drugs is limited by various physiological barriers. The first barrier met by nanocarriers is their rapid elimination from the blood circulation, resulting from a nonspecific uptake by the MPS. Then, prior to reaching tumor cells, the second barrier is their extravasation out of the blood vessel. Finally, they need to bind and release the entrapped drug, generally inside the tumor cells.

To overcome the rapid elimination from systemic circulation, stealth NPs were developed, and already show promising results (e.g., commercial formulations and new clinical trials in process). When stealth, both targeted and nontargeted NPs are generally able to arrive at the tumor vicinity via the EPR effect. However, to date, in the majority of studies, the use of active targeted nanocarriers has not provided evidence of increased tumor accumulation after intravenous injection (TABLE 2) [106,127], in spite of the presence of a specific ligand, except in the case of targeting specific cell types within the vasculature [128] (e.g., in order to reach the brain) [112]. However, there is an undeniable increase of therapeutic effect when the drug is loaded in active targeted NPs, owing to the enhancement of tumor cell internalization due to ligand–receptor interactions [106,129]. These active targeted NPs actually represent a very interesting therapeutic modality for the management of localized tumor when intratumoral injection is possible [118].

However, administration by intravenous route, despite a few encouraging results, still causes important problems. First, conjugation of active ligands may lead to an accelerated elimination of NPs by preventing their stealth properties, which, as described in this article, constitute the indispensable prerequisite. Thus, to add an active targeting agent, different parameters have to be considered (i.e., choice of ligands [130], method of coupling, concentration of ligands and control of ligand activity after coupling), as well as their influence on stealth properties. With passive targeted NPs, however, cell internalization could be prevented by a PEG layer, whereas active targeted counterparts can allow an increase in cellular uptake, which is an interesting advantage.

Accordingly, active targeting increases drug efficacy, and decreases systemic toxicity owing to drug release in normal cells. However, arrival at the drug's target site is not guaranteed, and drug efficacy is greatly dependent on intracellular trafficking and subsequent transport.

Actually, in spite of the different advantages expected with active targeting, currently no active targeted NPs are on the market, and only a few formulations are subjected to clinical experiments (TABLE 3). Thus, many papers in the literature describe the procedure and the *in vitro* characterization of formulation; nevertheless, few articles on activity after *in vivo* study have been published. Is it linked to the complexity of the formulation processes of these active targeted NPs, the expensive price of the ligands and effective scaling-up production, the difficulty to formulate these systems in large quantities for *in vivo* studies, or quite simply, to the lack of activity? Consequently, since passive targeted NPs are easier to produce, and owing to the low benefit supplied with active targeting in comparison with passive targeting, passive targeting NPs have been promoted [131] and, nowadays, constitute an interesting and promising field in nanotechnology development.

As a consequence, attempts to improve the effectiveness and safety of chemotherapy have brought about several tumor-targeted drug delivery strategies. From the benefits of PEG coating, the engineering of drug delivery nanoscale systems is moving in the direction of 'smart' devices, which are specially homed to, and responsive in, their target environment only [132,133]. For example, stealth NPs can possess a PEG hydrophilic surface, which is able to vanish upon arrival at the site of interest by the cleavage of a sensitive linkage via chemical stimuli, such as the presence of low pH or reducing agents, or enzymatic stimuli, such as proteases [134,135]. Nanocarriers could then be transformed to a more cell-interactive form, by restoring the possible interaction of the targeting moieties with the cell membrane receptors, once the tumor site is attained, and improving drug bioavailability [136]. For instance, Romberg *et al.* prepared long-circulating liposomes with an enzymatically degradable coating polymer that triggers cellular uptake of liposomes and/or release of the entrapped biologically active agent [88]. Such promising strategies offer not only a great increase in the intrinsic pharmacologic effect of drug-loaded in NPs for therapeutic success, but also prevention of interactions at nonspecific sites. In the near future, more safe and effective nanotechnology anticancer products for cancer therapy are expected, especially nanocarriers with multivalent properties (e.g., stealth, specific targeting, internalization and drug controlled release) (FIGURE 3 & 5).

Executive summary

Background

- Stealth nanoparticles still represent a promising generation of nanocarriers for cancer therapy.

Passive targeting

- Surface PEGylation has been successfully applied to various types of nanoparticles, such as liposomes, polymer nanoparticles, lipid nanoparticles and micelles. Polyethylene glycol coating confers to nanoparticles the stealth properties that limit nanoparticle elimination by the mononuclear phagocyte system in the bloodstream and allow tumor accumulation by the enhanced permeability and retention effect.
- Polyethylene glycol nanoparticles loaded with various anticancer drugs (e.g., taxanes, anthracyclines, metal-based drugs and camptothecin analogs) have shown promising results. These nanocarriers have entered clinical trials in humans and some are approved by the US FDA.
- Various parameters have to be taken into consideration, such as PEGylation process versus physicochemical characteristics of nanoparticles, tumor vasculature versus passive accumulation of nanoparticles, and safety versus efficacy of anticancer-loaded nanoparticles.

Active targeting

- Active targeted nanoparticles can be prepared with active ligands that confer selective cellular binding. Ligands can be antibodies, nucleic acid aptamers, peptides and small molecules, such as folic acid and sugar molecules.
- There is an undeniable increase of therapeutic effect when drug is loaded in active targeted nanoparticles thanks to the enhancement of tumor cell internalization due to the ligand/receptor interactions.
- In order to keep stealth properties with active targeted nanoparticles, various parameters have to be taken into consideration (e.g., choice of ligand, method of coupling and density of ligands). The control of ligand activity after coupling must be checked.

Conclusion

- Stealth nanocarriers can be designed to optimize chemotherapy after intravenous administration thanks to passive accumulation into tumor.
- Active strategy promises to be a powerful approach leading to effective drug delivery into tumor cells for the treatment of cancer, provided that stealth properties are kept.
- Beside the stealth and active properties, it would be interesting to design nanocarriers of new generation, being able to control drug release in the specific target. Thus, the multifunctional 'smart' devices represent the future nanomedicines.

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Publication de résultats n°3

Stratégie de ciblage passif par amélioration de la furtivité des LNC-FcdiOH

Ce chapitre envisage d'évaluer la chimiothérapie systémique des gliomes par injection intraveineuse des LNC-FcdiOH. Dans cette optique, le DSPE-mPEG2000 est utilisé pour conférer aux LNC des propriétés furtives, estimées par deux tests *in vitro* : l'activation du complément, mesurée par la méthode du CH50, et la capture par les macrophages humains THP-1 évaluée par une technique de cytométrie en flux. Le fluorochrome DiI est utilisé afin d'étudier les profils pharmacocinétiques des deux types de LNC chez le rat sain. Puis, ces LNC sont administrées par voie intraveineuse *in vivo* chez le rat porteur d'un gliosarcome sous-cutané ou intracérébral. Dans le modèle ectopique, le traitement est administré quand le volume tumoral atteint environ 76 mm^3. L'effet antitumoral est estimé par mesure de la taille de la tumeur au cours de l'expérimentation et de la masse tumorale à la fin de l'étude (25 jours après traitement). Dans le modèle orthotopique, les suspensions de LNC sont injectées à J6 après l'implantation des cellules 9L et l'appréciation de l'efficacité des traitements est évaluée par des études de survie des rats.

Soumise à Pharmaceutical Research

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

The present study deals with a passive-targeting strategy, via the enhanced permeability and retention effect following the systemic administration of lipid nanocapsules (LNCs) loaded with an organometallic tamoxifen derivative, Fc-diOH compound. In order to do this, the long chain of polyethylene glycol 2000 (DSPE-mPEG2000) was incorporated onto the surface of LNCs to provide them with long-circulating properties. Both LNCs and DSPE-mPEG2000-LNCs presented low *in vitro* complement activation and weak uptake by THP1 macrophages, but DSPE-mPEG2000-LNCs exhibited a prolonged half-life and an extended AUC in healthy rats. In a subcutaneous, 9L gliosarcoma rat model, a single intravenous injection of Fc-diOH-LNCs (400µL, 2.4mg/rat) considerably inhibited tumour growth when compared to the control. More interestingly, with the same dose, DSPE-mPEG2000-Fc-diOH-LNCs exhibited a strong antitumour effect by nearly eradicating the tumour by the end of the study. In an intracranial gliosarcoma model, treatment with DSPE-mPEG2000-Fc-diOH-LNCs and Fc-diOH-LNCs also statistically improved the median survival time (28 days and 27.5 days respectively) when compared to the untreated control (25 days). Results from this study demonstrate the interesting perspectives for the systemic treatment of glioma thanks to bio-organometallic chemotherapy via lipid nanocapsules.

Keywords: Fc-diOH, PEGylated nanoparticles, stealth properties, ectopic, orthotopic.

1. 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 ferrocenyl diphenol compound (Fc-diOH), has proved to be an effective cytostatic compound with an IC50 of about 0.5µM on 9L gliosarcoma cells, and highly-reduced toxicity was observed on astrocytes (IC50 = 50µM) [4]. This suggests that Fc-diOH 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 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 microemulsion 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 Fc-diOH was well encapsulated into the oily core of LNCs (Fc-diOH- LNCs) at high loading levels (6.5mg Fc-diOH per gram of LNC suspension, corresponding to 2% w/w dry weight) with a high encapsulation Fc-diOH efficiency (> 98%) [4]. Moreover, the *in vitro* cytostatic activity

of Fc-diOH was totally conserved after encapsulation. With respect to its *in vivo* antitumour activity, Fc-diOH-LNC treatment resulted in remarkable effects in an ectopic gliosarcoma rat model, following an intra-tumoural 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 as being 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(polyethylene-glycol)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 Fc-diOH-LNCs versus DSPE-mPEG2000-Fc-diOH-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).

2. Materials and methods

2.1. Materials

Ferrocenyl diphenol compound (2-ferrocenyl-1,1-bis(4-hydroxyphenyl)-but-1-ene) named Fc-diOH 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) = 2,805g/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).

2.2. 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 160-180g. Animal care was carried out in strict accordance with French Ministry of Agriculture regulations.

2.3. 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 and followed by a sudden dilution with 28.5% v/v of cold water added to the mixture at 70-75°C.

To load the anticancer agent into the oily core of LNCs, Fc-diOH was firstly 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 Fc-diOH-LNCs at a high drug concentration of 6.5mg/g (2% w/w dry weight).

Fluorescent LNCs were obtained by labelling LNCs with DiI fluorochrome (emission wavelength = 549nm; excitation wavelength = 565nm). The preparation was performed as previously described [20]. Briefly, DiI was dissolved in acetone at 3mg/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.

2.4. Post-insertion of DSPE-mPEG2000 onto LNC surfaces

DSPE-mPEG2000 was incorporated onto the surface of LNCs at the concentration of 10mM by the post-insertion technique as previously described [13]. Briefly, DSPE-mPEG2000 was firstly dispersed in the water (1/10 total final volume) at 60^0C for 15 min. to form micelles. Preformed LNC suspension and DSPE-mPEG2000 micelles were thereafter co-incubated for 2h at 60^0C. The mixture was vortexed every 15 min., and finally quenched in an ice bath for 1 minute.

2.5. 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 25^0C. 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).

2.6. *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 [21], 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. Nanoparticle surface areas were calculated as previously described [22]. 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.

2.7. *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 37^0C in ATCC recommended medium. Cells were cultured in the medium containing 1:1 solution of ATCC recommended medium and 200nM Phorbol 12-myristate 13-acetate (PMA, Sigma, Saint-Quentin Fallavier, France) for 48h to allow adherence and differentiation [23]. The medium was then aspired and the cells were subsequently incubated in a new medium for an additional 24h prior to uptake studies. The cells were harvested and counted using Trypan blue exclusion assay with a haemacytometer. Cells (0.6 x 10^6/mL) were then placed on a 24-well plate for 24h.

Dil-labelled LNC suspensions were diluted in MiliQ water to obtain a concentration of particles in suspension of 16.5mg/g. 24h after culture, the culture medium was totally removed and cells were incubated at 4^0C or 37^0C for 1.5h with 50µL of previously-prepared LNC suspensions and 500µL 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.

2.8. *In vivo* pharmacokinetic study

400µL 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,

5mL, Becton Dickinson France SAS, Le Pont-De-Claix, France). 150µL 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 544nm with an excitation wavelength of 590nm by a Fluoroscan (Ascent FL, Thermo Fisher Scientific, Cergy-Pontoise, France). Plasma residual fluorescence was measured from the supernatant of centrifuged blood taken from 3 rats receiving 400µL of a physiological saline solution. Fluorescence was expressed in fluorescence units (FU) and was calculated as: $FU_{sample} - FU_{residue}$. 100% 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 24h.

2.9. *In vivo* antitumour efficacy study

2.9.1. 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% 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). On the day of implantation,

cells were trypsinised and re-suspended into minimal essential medium (EMEM), without FCS or antibiotics, to the final desired concentration.

2.9.2. Subcutaneous gliosarcoma model and therapy schedule

Animals were manipulated under isoflurane/oxygen anaesthesia. After shaving and disinfection, rats were subcutaneously implanted with $1.5x10^6$ 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: $V = (\pi/6) \times (width)^2 \times (length)$.

When tumours reached a calculated average volume of approximately $76mm^3$, the rats were randomised into 4 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 (400µL) via the lateral tail vein as follows: physiological saline solution (0.9% NaCl), blank LNCs, Fc-diOH-LNCs (2.4mg/rat) and DSPE-mPEG2000-Fc-diOH-LNCs (2.4mg/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 were considered as significant with $P < 0.05$.

2.9.3. Intracranial gliosarcoma model and therapy schedule

The animals were anaesthetised by an intraperitoneal injection of a mixture of 1:1 solution of ketamine (100mg/kg body weight) (Clorketam®, Vétoquinol, Lure, France) and xylazine (20mg/kg body weight) (Rompun®, Bayer, Puteaux, France). 10 microlitres of 10^3 9L cell suspension were stereotaxically implanted for 5 min. into the rat striata using a 10µl syringe

(Hamilton® glass syringe 700 series RN) with a 32G needle (Hamilton®) [8]. The cannula coordinates were 1mm posterior from the bregma, 3mm lateral from the sagittal suture and 5mm below the dura (with the incisor bar set at 0mm).

6 days after 9L cell implantation, the rats were anaesthetised by isoflurane/oxygen inhalation. Rats were randomly treated by intravenous injection (400µL) with blank LNCs, Fc-diOH-LNCs (2.4mg/rat), or DSPE-mPEG2000-Fc-diOH-LNCs (2.4mg/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:

$$\% \text{ IST} = [\text{Median}_T (\text{Mean}_T) - \text{Median}_C (\text{Mean}_C)]/ \text{Median}_C (\text{Mean}_C)$$

where $\text{Median}_T/\text{Mean}_T$ was the median/mean of survival time of the treated group and $\text{Median}_C/\text{Mean}_C$ was the median/mean of survival time of the control group.

3. Results

3.1. Preparation and characterisation of LNCs

The physicochemical properties of the different kinds of LNCs are presented in Table 1. The size distribution of LNCs was unimodal (PdI < 0.1). Blank LNCs presented a median diameter of 47.92 ± 0.93nm with a slightly negative zeta potential (-6.51 ± 1.01mV). The loading with Fc-diOH as well as the DiI fluorochrome into the oily core of LNCs did not affect the particle size or the zeta potential. The determination of the drug loading by spectrophotometry at 450nm after dissolving LNCs in a mixture of 22/67/11 (v/v/v) acetone/THF/water solution (as previously described [4]) showed that Fc-diOH was well encapsulated in the LNCs at a high drug-loading capacity (6.44 ± 0.08mg/g) and 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 -22.5mV) owing to the formation of dipoles between PEG molecules and water, as previously described [24].

Table 1. 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
Fc-diOH-LNCs	46.07 ± 0.11	0.038 ± 0.011	-6.96 ± 0.67
DSPE-PEG-Fc-diOH-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

3.2. 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 Figure 1, all types of tested LNCs, including blank LNCs, Fc-diOH-LNCs, DSPE-mPEG2000-Fc-diOH-LNCs, demonstrated weak complement activation. Indeed, at a total surface area of approximately 1,000cm^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 300-400cm^2 [13, 21]. Among the 3 types of tested LNCs, the slight increase of the CH50 value of DSPE-mPEG2000-Fc-diOH-LNCs could be attributed to their increase in mean particle size, as already observed [21, 25].

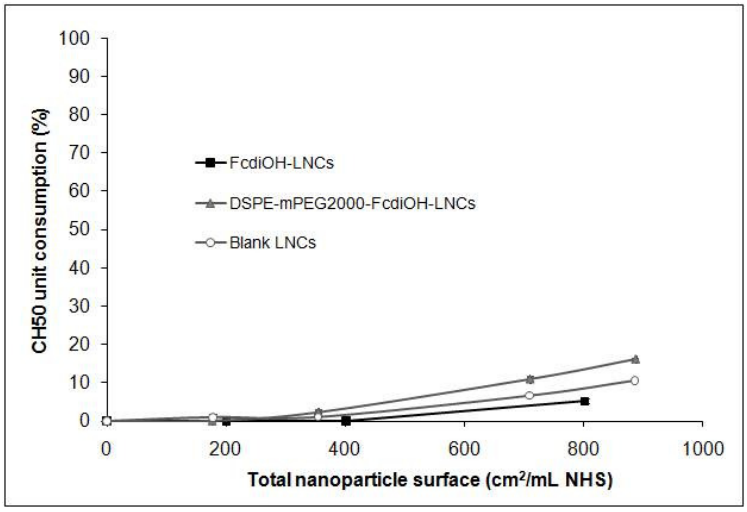

Figure 1. Complement activation of blank LNCs, Fc-diOH-LNCs and DSPE-mPEG2000-Fc-diOH-LNCs expressed by % consumption of CH50 unit at 37^{0}C in function of the nanoparticle surface area. Results are represented as mean ± SEM.

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 4^0C or 37^0C. The macrophage uptake of particles at 37^0C represents the total uptake, including the adsorption of nanoparticles onto the cell surface and the active cellular endocytosis. At 4^0C, 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. Moreover, the macrophage uptake of LNCs by THP-1 cells at 37^0C was not significantly different from this at 4^0C. This suggested a low *in vitro* phagocytosis of tested LNCs by macrophage cells.

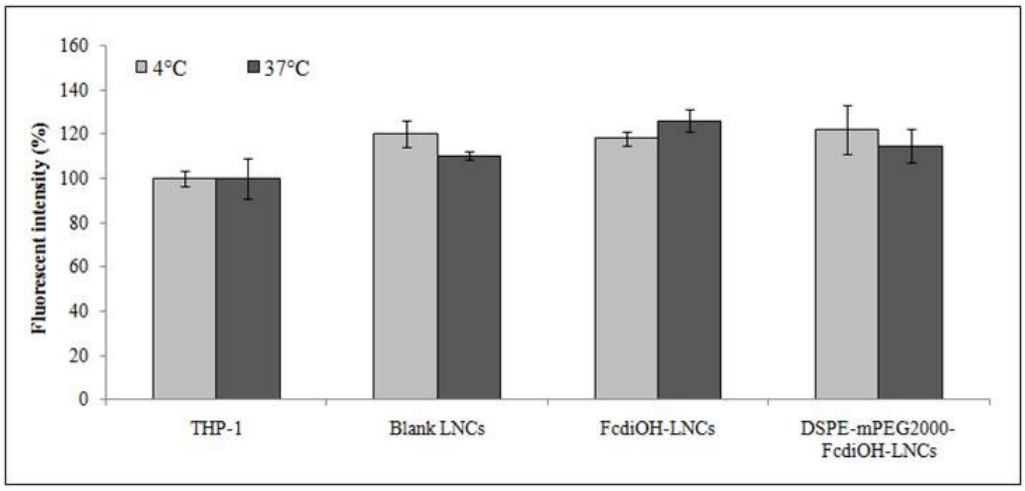

Figure 2. Quantification of *in vitro* macrophage uptake of blank LNCs, Fc-diOH-LNCs and DSPE-mPEG2000-Fc-diOH-LNCs. Fluorescent intensity was measured 24h after incubation of DiI-labelled LNCs with THP-1 cells. The residual fluorescence of THP-1 cells alone was scaled to 100%. Results are represented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

3.3. Pharmacokinetics of LNCs in rats

As shown in the results of physicochemical characteristics, the loading with either Fc-diOH 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 LNCs (DiI-LNCs) and PEGylated LNCs (DSPE-mPEG2000-DiI-LNCs) which were considered as being representative of Fc-diOH-LNCs and DSPE-mPEG2000-Fc-diOH-LNCs.

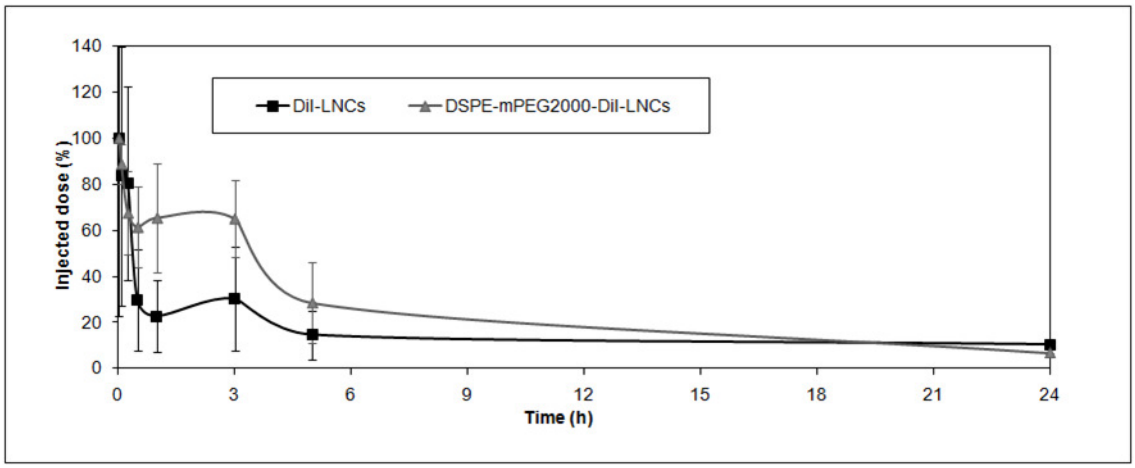

Figure 3. Plasma concentration-time profile of conventional DiI-LNCs and DSPE-mPEG2000-DiI-LNCs during 24h after a single, intravenous injection into healthy female Fisher rats. Data are represented as mean ± SEM.

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, 24h 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 3h post-injection. Finally, approximately 10% of the injected dose of both DiI-LNCs and DSPE-mPEG2000-DiI-LNCs remained in the blood 24h post-injection.

By calculating with Kinetica 4.1.1 software, the estimated half-life value for DiI-LNCs was 1.95h with AUC_{last} of 381 (% injected dose/h). DSPE-mPEG2000-DiI-LNCs exhibited a

prolonged half-life of 7.82h with an extended AUC of 623 (% injected dose/h). This result is in accordance with 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, 21].

3.4. Anti-tumour activity of Fc-diOH-LNCs on a subcutaneous gliosarcoma model

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

The results presented in Figure 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 3,000mm^3 and 2,300mm^3, respectively. These corresponded to a tumour mass of 2,400 and 1,850mg, respectively at Day 25. The intravenous administration with Fc-diOH-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 1,300mm^3 with a mass of 1,050mg. On the contrary, DSPE-mPEG2000-Fc-diOH-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 33mg by the end of the study.

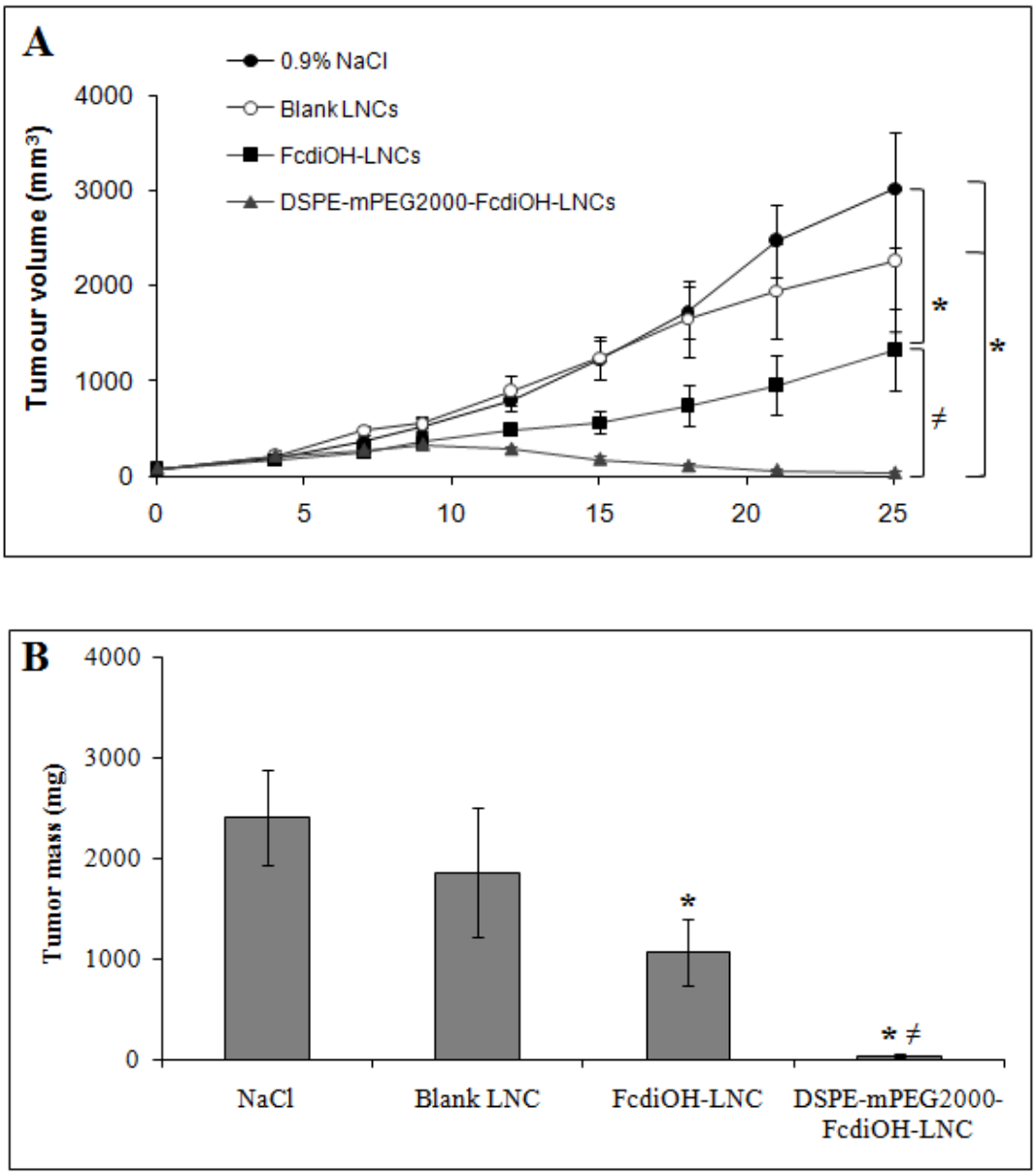

Figure 4. Anti-tumoural effect of Fc-diOH-LNCs and DSPE-mPEG2000-Fc-diOH-LNCs after a single intravenous treatment as compared to control groups receiving 0.9% NaCl solution or blank LNCs in a subcutaneous 9L gliosarcoma rat model. Treatment was initiated when the tumour volume reached about 76mm^3 (Day 0). Tumour growth (A) was estimated by measuring twice weekly the length and width of tumours with callipers and 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 Fc-diOH-LNC treatment, respectively.

3.5. Anti-tumour activity of Fc-diOH-LNCs on an intracranial gliosarcoma model

The promising results of anti-tumour activity of Fc-diOH-LNCs and DSPE-mPEG2000-Fc-diOH-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 Fc-diOH-LNCs and DSPE-mPEG2000-Fc-diOH-LNCs at the same dose used in the subcutaneous 9L gliosarcoma model (400µL, 2.4mg/rat).

Table 2. Survival time of intracranial, 9L gliosarcoma-bearing rats that received an intravenous injection of blank LNCs, Fc-diOH-LNCs or DSPE-mPEG2000-Fc-diOH-LNCs at Day 6 after cell implantation, compared to an untreated control group.

Treatment	n	Survival time (days)			% IST		P *vs* untreated control
		Range	Median	Mean ± SD	Median	Mean	
DSPE-mPEG2000-Fc-diOH-LNCs (2.4mg/rat)	8	27-32	28	28.75 ± 1.58	12	14.41	0.0001
Fc-diOH-LNCs (2.4mg/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.

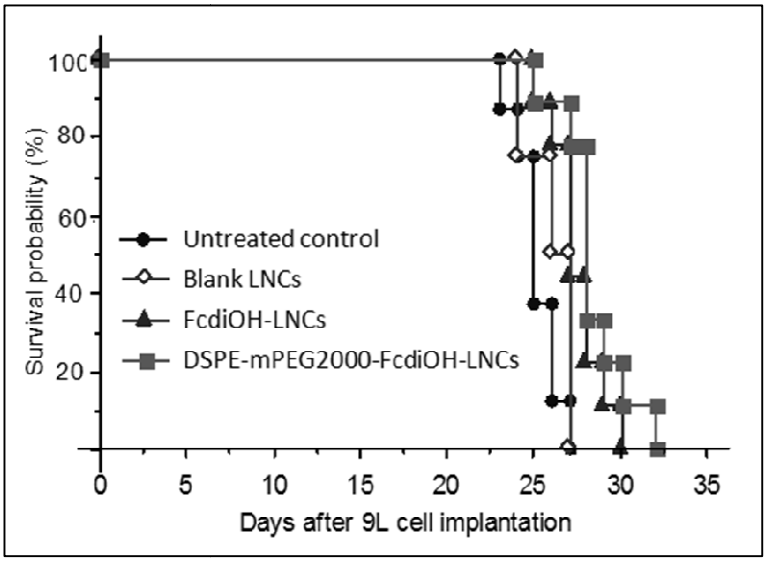

Figure 5. Kaplan-Meier curves plot the survival times of experimental rats with an intracranial, 9L gliosarcoma model. 9L gliosarcoma cells were implanted intracranially into the striata of rats, followed by the intravenous administration of blank LNCs, Fc-diOH-LNCs or DSPE-mPEG2000-Fc-diOH-LNCs at Day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

The survival data of the experimental rats are summarised in Table 2 and Kaplan–Meier survival plots are shown in Figure 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 was 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 Fc-diOH-LNCs was statistically

different from the untreated control group. Finally, DSPE-mPEG2000-Fc-diOH-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 Fc-diOH-LNC treated group ($P = 0.1914$).

4. 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 [26-31]. 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 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 [32]. Results from *in vitro* studies showed that all kinds of tested LNCs, including blank LNCs, Fc-diOH-LNCs and DSPE-mPEG2000-Fc-diOH-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 Fc-diOH 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 Fc-diOH-LNCs significantly reduced the tumour volume as well as tumour mass, showing the *in vivo* effectiveness of Fc-diOH in inhibiting tumour growth. The profile of tumour volume versus time was comparable to that of the intratumoural injection of Fc-diOH-LNCs on the same ectopic gliosarcoma model in rats as previously observed [4]. The remarkable thing in the present work is that DSPE-mPEG2000-Fc-diOH-LNC treatment nearly eradicated subcutaneous tumours following a single intravenous injection. This indicates, on one hand, the accumulation of DSPE-mPEG2000-Fc-diOH-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 anti-tumoural activity of Fc-diOH was strengthened. Indeed, a large number of studies in the literature have usually dealt with repeated injections [33-36] or a combination treatment [37] to achieve significant efficacy.

In our more clinically-relevant, orthotopic gliosarcoma model, the single intravenous administration of Fc-diOH-LNCs and DSPE-mPEG2000-Fc-diOH-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 Fc-diOH-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 [38-39]. 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 8h 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 [40]. For example, pegylated liposomal doxorubicin (0.1mg/mL, 20µL) delivered locally by CED injection, significantly prolonged the survival time of intracranial U251MG human glioblastoma-bearing rats (median survival time of 84.5 days) but failed in treatment with a three-weekly, intravenous administration regimen at the maximum tolerated dose of doxorubicin (17mg/kg) (median survival time of 45.5 versus 47.5 days for the control group) [16].

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 [41-43], 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 Fc-diOH 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.

5. 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 Fc-diOH, 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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Publication de résultats n°4

Stratégie de ciblage actif par greffage d'un peptide à la surface des LNC-FcdiOH

Ce dernier chapitre, présenté sous forme de communication courte, consiste en une étude préliminaire de ciblage actif suite au greffage d'un peptide, le NFL-TBS, à la surface des LNC-FcdiOH. Le peptide NFL-TBS, aux propriétés supposées de peptide internalisant, devrait permettre une meilleure entrée des peptide-LNC-FcdiOH dans les cellules, si l'accumulation sur le site est bien effective. Tout d'abord, la viabilité des cellules de gliosarcome 9L après différents traitements est mesurée à l'aide d'un test au MTS (3-(4,5-diméthylthiazol-2-yl)-5-(3-carboxyméthoxyphényl)-2-(4-sulfophényl)-2H-tétrazolium).
L'efficacité antitumorale *in vivo* de ces nanovecteurs est ensuite évaluée par des études de survie des rats porteurs d'un gliosarcome 9L intracérébral après injection en CED ou par voie intra-carotidienne.

Soumise à European Journal of Pharmaceutics and Biopharmaceutics

An active targeting strategy through ferrociphenol-loaded lipid nanocapsules for the treatment of intracranial 9L rat gliosarcoma

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Abstract

In this study, internalising cell peptide (NFL-TBS peptide) that specifically interacts with tubulin-binding sites was incorporated onto the surface of lipid nanocapsules (LNCs) through an active targeting strategy to favour the internalisation of LNCs into the tumour cells. Blank peptide-LNCs had no effect on the 9L cell *in vitro* viability while they showed a strong cytotoxicity when loaded with the ferrociphenol compound (FcdiOH). The incorporation of peptide allowed an enhanced cytotoxicity of FcdiOH-LNCs. On a gliosarcoma intracranial rat model, CED treatment with peptide-FcdiOH-LNCs seems to be toxic due to 50% of animal mortality, resulting probably from an overdose problem of administered drug. Interestingly, intra-carotid administration of these LNCs led to an ameliorated survival time of treated rats, with a mean survival time of 28 days as compared to 25 days for untreated control group, and one rat surviving until 44 days. This primary study demonstrated the benefit of such a peptide as an active ligand for drug delivery to the brain tumours.

Keywords: active ligand, NFL-TBS peptide, intra-carotid injection, CED, LNC, brain tumour.

1. Introduction

Nanotechnology emerges nowadays as an interesting field in engineering and designing new systems for drug delivery. It can provide medical and pharmaceutical benefits, especially in oncology, because it enables the control of drug characteristics such as solubility, bioavailability, vascular circulation time, and specific site-targeted delivery [1-2]. Our laboratory developed and patented a novel nanoscale system, the so-called lipid nanocapsules (LNCs), whose formulation is based on the phase-inversion phenomenon of a microemulsion [3]. They possess an oily core, corresponding to medium-chain triglycerides surrounded by a tensioactive, cohesive membrane made by a mixture of lecithin and a poly(ethylene glycol) PEG 660 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 [4]. Further modifications of their surface by coating with longer PEG chains in a context of a passive targeting strategy, or by incorporating active ligand through an active targeting strategy, are feasible in order to improve the drug delivery to the site of interest, i.e. cancer tissues [5-6].

The compound 2-ferrocenyl-1,1-bis(4-hydroxyphenyl)-but-1-ene, or ferrociphenol (FcdiOH) is one of promising anticancer drug candidates in the series of ferrocifen-type by grafting a ferrocenyl unit onto a tamoxifen skeleton [7]. It was shown to be effective on hormone-dependent and hormone-independent breast cancer cell lines [8] as well as specifically towards 9L rat gliosarcoma cells but not to astrocytes [9]. In addition, this *in vitro* effect was totally recovered after its loading into the oily core of LNCs (FcdiOH-LNCs). Moreover, intra-tumour injections of FcdiOH-LNCs resulted in remarkable effects in the heterotopic and orthotopic 9L gliosarcoma rat models [9-10].

Recently, Bocquet et al. (2009) showed that a peptide derived from the light neurofilament subunit (NFLs) which specifically interacts with tubulin-binding sites (TBSs), could enter in multiple cancer cell lines leading to disruption of their microtubule network and reduction of their proliferation [11].

Taking these advantages into consideration, the present study dealt with an active targeting by using NFL-TBS peptide that was incorporated onto the surface of FcdiOH-LNCs. The efficacy of these nanovectors was firstly evaluated on cultured 9L gliosarcoma cells, and then, on intracranial gliosarcoma-bearing rats treated by means of convection-enhanced delivery (CED) or an intra-carotid injection.

2. Materials and methods

2.1. Chemical materials

Ferrociphenol was prepared by a McMurry coupling reaction (Jaouen *et al.*, 2000b). The lipophilic Labrafac® CC (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).

2.2. Tumour cell line and culture

Rat 9L gliosarcoma cells were obtained from the European Collection of Cell Culture (Salisbury, UK, N°94110705). The cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) with glucose and L-

glutamine (BioWhittaker, Verviers, Belgium) supplied with 10% foetal calf serum (FCS) (BioWhittaker) and 1% antibiotic and antimycotic solution (Sigma, Saint-Quentin Fallavier, France).

2.3. Animals

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

2.4. Preparation of the LNCs

2.4.1. Anticancer agent-loaded LNCs

LNCs were prepared according to the original described procedure (Heurtault *et al.*, 2002). Briefly, the preparation process involved 2 steps. Step I consisted of mixing all the components (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)) 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 minutes.

To load the anticancer agent to the oily core of LNCs, FcdiOH was firstly dissolved in Labrafac® under ultrasound at 4% (w/w) for 1.5 hours and the resulting lipophilic phase was afterwards mixed with other components, as described above, to prepare a suspension of FcdiOH-LNCs at a concentration of 6.5 mg of FcdiOH per mL of LNC suspension. Final LNC suspensions were filtered through a Minisart 0.2 µm filter (Sartorius).

2.4.2. Incorporation of peptide onto the LNC surface

Prior to incubation with peptides, LNC suspension was passed through a PD-10 sephadex column (Amersham Biosciences Europe, Orsay, France) and then concentrated by centrifugations by using a Millipore Amicon 100 kDa centrifugal filter device (Millipore, St Quentin-Yvelines, France). Resulting LNC suspension (1 mL) was then incubated for 24 h with 0.369 µL of 1 mM peptide solution prepared in MiliQ water at room temperature under slow magnetic rotation.

2.5. LNC characterisation

The LNCs were diluted 1:100 (v/v) in deionised water. 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).

2.6. *In vitro* cell viability

A suspension of 9L cells ($2x10^4$ 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 increasing concentrations (0.01 to 100 µmol/L) from various formulations. The free FcdiOH (non-encapsulated FcdiOH) solubilised in absolute ethanol at a concentration of 0.1M was diluted at 1:1000 in the culture media to obtain the highest concentration (100 µmol/L). Different LNC suspensions were diluted in the culture media to the 100 µmol/L concentration. The other low concentrations were obtained by 1:10 cascade dilution from the 100 µmol/L concentration. Blank LNCs and blank peptide-LNCs served as controls with the same dilution procedure being made for corresponding drug-loaded LNCs.

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. MTS solution (100 µl) was added to each well, and the plates were incubated at 37°C for 3h. 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 present 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 mixture of ketamine (100 mg/ml) (Clorketam®, Vétoquinol, Lure, France) and xylazine (20 mg/ml) (Rompun®, Bayer, Puteaux, France). Ten microlitres of 10^5 9L cells/mL were injected stereotaxically into the right rat striatum by using a 32G needle (Hamilton®) fitted to a 10 µl Hamilton® syringe, as previously described [12], with the cannula coordinates as follows: 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).

On Day 6 after 9L cell implantation, rats were injected by means of CED or intra-carotid injection of different LNC formulations. The control group did not receive any treatment.

2.7.2. CED procedure

Tumour-bearing-rats were anaesthetised by an intra-peritoneal injection of 1.5 ml/kg of a 2:1 mixture of ketamine and xylazine. The 32G needle (Hamilton®) fitted to a 10 µl Hamilton® syringe which was connected, through a cannula, to a 100 µl Hamilton syringe 22G containing the product (Harvard Apparatus, Les Ulis, France) was placed in the same coordinates of 9L cell implantation. Sixty microlitres (60 µL) of LNC suspension (0.33 mg of

FcdiOH/rat) were injected by CED which was performed with a pump PHD 2,000 infusion (Harvard Apparatus, Les Ulis, France) by controlling a 0.5 µl/min rate for 2h as previously described [12].

2.7.3. Intra-carotid treatment

Tumour-bearing rats were treated by 400 µL of intra-carotid injection of peptide-FcdiOH-LNCs (2.2 mg of FcdiOH/rat). For this purpose, rats were anaesthetised by an intra-peritoneal injection of 1.0-1.5 ml/kg of a 1:1 solution of ketamine and xylazine. The middle neck skin was shaved and incised. The right common carotid was exposed and ligated. A PE10 polyethylene catheter (BD Intramedic™ Polyethylene Tubing, Becton Dickinson, USA) was inserted retrogradely through a small arteriotomy. LNC suspensions (400µL) were injected into each rat. The catheter was then removed and the carotid artery was ligated.

Animals were weighted every 6 days. Rats were sacrificed in a CO_2 chamber when they lost 20 % of body weight and/or as soon as they presented seizure, a hunched posture, or haemorrhaging around the eyes, mouth and nose. 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.

The statistical analysis was 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 $P < 0.05$. The different treatment groups were compared in terms of median and mean survival time (days).

3. Results and discussion

3.1. Physicochemical properties of LNC suspensions

Blank LNCs presented an average hydrodynamic diameter of 47.58 ± 0.84 nm and a very narrow size dispersion (PdI = 0.041 ± 0.011). As described in a previous study relating the good encapsulation of FcdiOH into the oily core of LNCs [9], its loading did not alter the particle size and the PdI (44.97 ± 2.47 nm and 0.054 ± 0.017, respectively). The adsorption of NFL-TBS peptide onto the LNC surface appeared to have no effect on the particle size (45.74 ± 1.40 nm), but increased significantly the PdI (0.199 ± 0.011). The determination of the drug loading by spectrophotometry at 450 nm after dissolving LNC suspension in a mixture of 22/67/11 (v/v/v) acetone/THF/water solution (as previously described [4]) showed that the drug payload in suspension of peptide-FcdiOH-LNCs was about 5.54 ± 0.06 mg/mL.

3.2. Cytotoxicity *in vitro*

The MTS assay was used to determine the *in vitro* cytotoxicity of different treatments against 9L gliosarcoma cells after exposure with a cascade concentration range of FcdiOH (0.01 to 100 μM). The cell survival profiles of various kinds of LNCs and free FcdiOH in absolute ethanol were illustrated in the Figure 1. Blank LNCs as well as blank peptide-LNCs seems to be tolerated by 9L cells, except the highest concentration of blank LNCs. At the low concentrations (0.01 to 0.1 μM), free FcdiOH as well as its encapsulated forms did not alter the cell growth (over 90%) whereas cell survival was dramatically decreased (below 20%) at concentration of 10 μM, resulting in an IC_{50} of about 1.5 and 1 μM for free FcdiOH and FcdiOH-LNCs, respectively. Thus, the encapsulation of FcdiOH into the oily core of LNCs did not compromise the activity of FcdiOH, as previously reported [9]. Interestingly, the incorporation of peptide seems to enhance the *in vitro* toxicity of FcdiOH-LNCs towards 9L cells with an IC_{50} of about 0.7 μM for FcdiOH-LNCs.

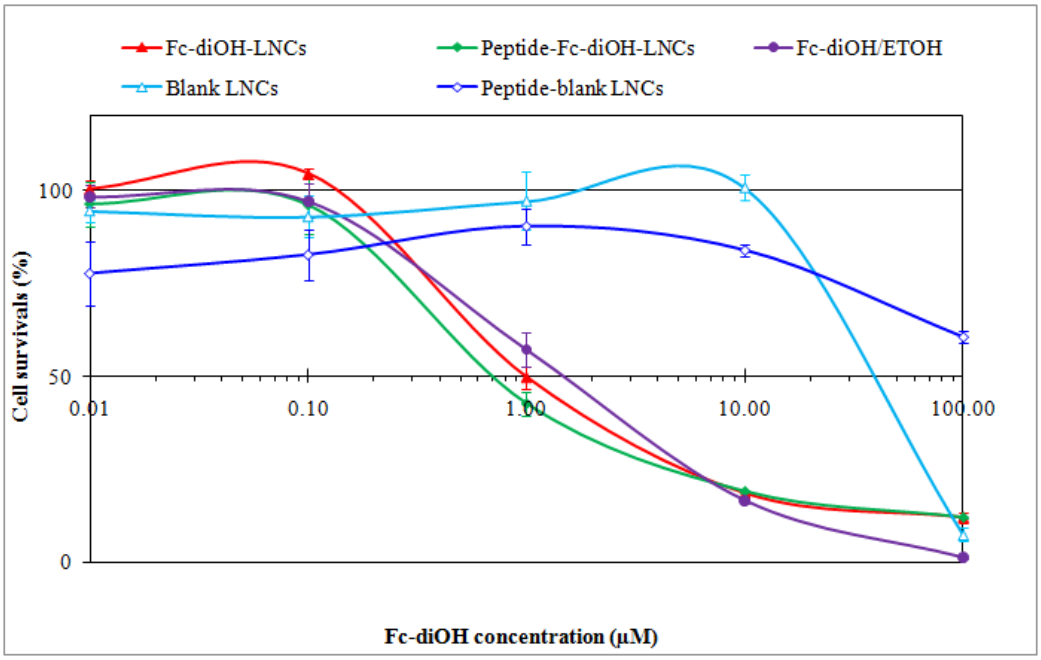

Figure 1: Cell survival of 9L gliosarcoma cells after 72h exposure to different treatments with free FcdiOH in ethanol or with various kinds of LNCs at different concentrations of drug (0.01 to 100 µM).

3.3. Survival studies

Taking advantage of local/locoregional administrations for drug delivery to the brain tumour that were previously highlighted [13], in this study, peptide-FcdiOH-LNCs were injected on Day 6 after cell implantation by means of CED or intra-carotid injection. The animal survival times were plotted on Kaplan-Meier curves (Figure 2).

The administration of peptide-FcdiOH-LNCs by CED caused 50% of treated rats which died 2 days after infusion. Moreover, the remaining rats presented a median survival time of 21 days against 25 days of untreated control rats. This result revealed the toxicity of the treatment towards the animal brain. A hypothesis can be brought forwards to explain this negative result. In fact, the presence of peptide on the LNC surface should potentially favour

the internalisation of nanovectors into the cancer cells that enhance the efficacy of LNC-FcdiOH, as observed above in *in vitro* study. In the case of FcdiOH, a cytostatic compound, an overdose of drug could lead to a cytotoxicity resulting in the cell death by necrosis. This pro-inflammatory form can be followed by the liberation of intracellular proteins and nucleic acids in extracellular space [14]. These mediators are responsible to trigger inflammatory response and other negative effects towards adjacent heathy cells [15-16].

With respect to the intra-carotid administration, treatment with peptide-FcdiOH-LNCs remarkably increased the survival time of rats with a median of 28 days and a mean of 29.5 days (Table 1). Moreover, one rat survived until 44 days after 9L cell implantation. This is an exceptional case for chemotherapy alone with FcdiOH-LNCs, showing the great potential of this approach for the glioma treatment. However, the sample size being still low, further investigation will be taken into consideration in order to confirm these interesting results.

Table 1. Survival time of intracranial 9L gliosarcoma-bearing rats treated with peptide-FcdiOH-LNCs by means of CED or an intra-carotid injection, compared to an untreated control group.

Treatment	n	Dose of FcdiOH (mg/rat)	Survival time (days)		
			Range	Median	Mean ± SD
Intra-carotid	6	2.2	24-44	28	29.5 ± 7.5
CED	3	0.33	20-22	21	21.0 ± 1.0
Untreated control	8	---	23-27	25	25.1 ± 1.3

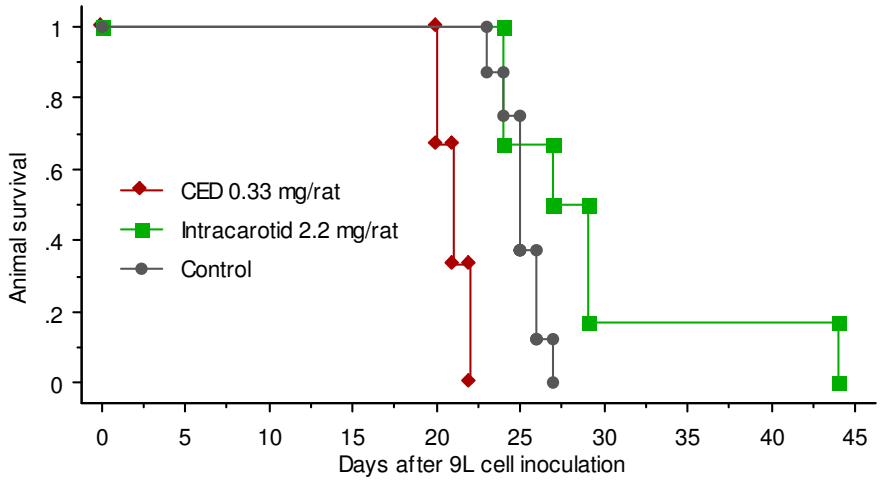

Figure 2: Kaplan-Meier survival plots for 9L gliosarcoma-bearing rats treated with peptide-FcdiOH-LNCs by means of CED or intra-carotid injection.

4. Conclusion

Primary results from this study revealed the usefullness of active targeting through lipid nanocapsules in drug delivery to the tumour cells. This also highlighted the benefit of such a cell-specific internalising peptide.

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DISCUSSION GENERALE

Malgré les améliorations considérables obtenues récemment dans le diagnostic et le traitement des gliomes, le pronostic de survie pour ce type de tumeur reste sombre depuis plusieurs décennies, en raison de sa localisation et de son caractère agressif et infiltrant [1]. Les possibilités thérapeutiques du traitement des gliomes intracérébraux sont restreintes et le rôle de la chimiothérapie reste marginal.

L'administration locale directement dans le parenchyme cérébral offre la possibilité de s'affranchir de l'obstacle de la BHE et de délivrer des agents anticancéreux à une concentration importante au sein des tissus tumoraux. Dans ce cadre, la technique CED (convection-enhanced delivery) représente une approche prometteuse pour une délivrance locale des LNC dans le cerveau. Elle permet d'administrer un volume plus important que la simple stéréotaxie (60µL au lieu de 10µL) et d'augmenter la distribution des LNC dans le striatum qui concerne alors totalement l'hémisphère injecté [2]. Les techniques d'administration locorégionales et systémiques permettent également d'atteindre la tumeur intracérébrale mais, dans ce cas, une attention particulière doit être portée au recouvrement de surface des nanovecteurs : greffage de longues chaines de PEG dans le cas d'un ciblage passif [3] ou de ligands spécifiques lors d'un ciblage actif [4].

Ce travail de thèse avait pour objectif d'optimiser l'efficacité de la chimiothérapie via les LNC chargées en ferrociphénol (FcdiOH), une molécule organométallique innovante [5], dans le traitement des gliomes en faisant varier :

- le type de formulation des LNC : LNC-FcdiOH classiques ou concentrées, ajustées ou non (osmolarité et pH), pégylées ou non (DSPE-mPEG2000), greffées ou non (anticorps monoclonal OX26 ou peptide d'internalisation cellulaire NFL-TBS) (Figure 1).
- la voie d'administration : locale par stéréotaxie/CED, locorégionale par injection intra-carotidienne et systémique par voie intraveineuse.

Dans cette partie, nous reprenons les points les plus importants concernant les résultats obtenus et, en complément, décrivons certains résultats non publiés permettant d'approfondir la discussion.

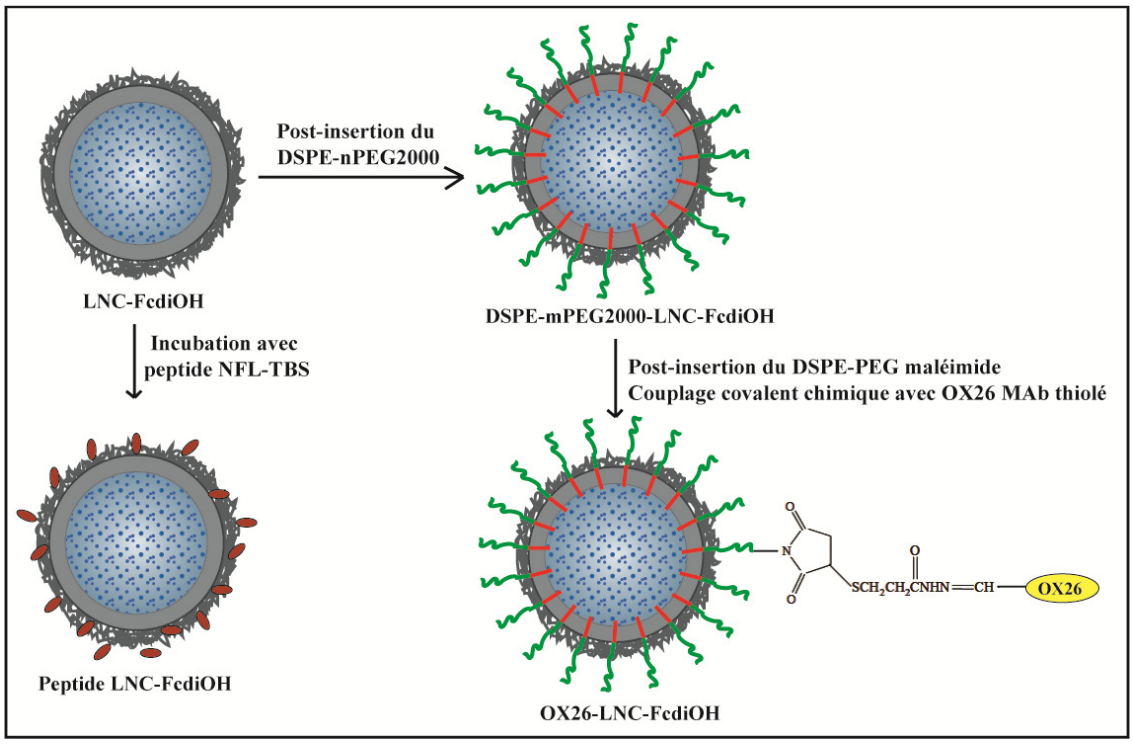


Figure 1 : Représentation schématique des différents types de LNC-FcdiOH testés

1. Modèles animaux utilisés

Deux modèles de gliosarcome 9L ont été établis sur des rats Fisher femelles afin d'évaluer l'effet antitumoral des LNC-FcdiOH : un modèle ectopique (tumeur sous-cutanée, dans le flanc de l'animal) et un modèle orthotopique (tumeur intracérébrale, implantée dans le striatum). La lignée cellulaire 9L est l'une des plus utilisées parmi les modèles de tumeurs cérébrales chez l'animal. Elle est produite par des multi-injections hebdomadaires de N-méthylnitrosourée (MNU) (5 mg/kg) pendant 26 semaines chez le rat CD Fisher [6-7]. La tumeur est clonée et présente initialement des caractéristiques gliales ; cependant, après un certain nombre de passages, ces caractéristiques se modifient pour prendre l'apparence d'un gliosarcome 9L [8].

Le modèle de gliosarcome sous-cutané a été testé dans un premier temps car il est facile à implanter ; il permet d'évaluer l'efficacité du traitement par simple mesure de la taille tumorale. Les cellules 9L sont injectées à raison de $1.5x10^6$ cellules dans le flanc de l'animal.

La taille de la tumeur (longueur et largeur) est régulièrement mesurée par un pied à coulisse. Le volume tumoral est calculé par la formule ellipsoïdale mathématique : $V = (\pi/6) \times (\text{Largeur})^2 \times (\text{Longueur})$. Le traitement est effectué quand le volume tumoral atteint environ 76 mm^3, en fonction des données de la littérature. Les animaux sont sacrifiés 25 jours après le traitement et la masse tumorale est prélevée et pesée. Cependant, ce modèle sous-cutané est très éloigné du modèle intracérébral qui possède des caractéristiques uniques dues à sa localisation particulière et à la présence de la BHE.

L'étude du modèle orthotopique de gliosarcome est donc indispensable dans le cas du traitement des gliomes. Pour ce faire, 10^3 cellules 9L sont implantées directement dans le striatum des rats grâce à un cadre de stéréotaxie (coordonnées : 1 mm postérieur du Bregma, 3 mm latéral de la suture sagittale et 5 mm sous le crâne). Dans ce cas, l'efficacité du traitement est évaluée par l'analyse de la survie des rats. Conformément aux normes éthiques, les animaux sont sacrifiés quand ils perdent 20% de leur poids et/ou quand ils présentent des signes caractéristiques de détresse et de douleur dus à l'augmentation de la taille de la tumeur intracérébrale : activité réduite, dos voûté, poils ébouriffés et hémorragie autour des yeux, de la bouche et du nez. Le jour de mort est considéré comme celui du lendemain du jour de sacrifice et les temps de survie des rats sont représentés sur une courbe de Kaplan-Meier.

Les différentes voies d'administration ainsi que les volumes injectés dans les deux modèles sont rassemblés dans le tableau récapitulatif suivant (Tableau 1) :

Tableau 1 : Voies d'administration et volumes d'injection étudiés dans les modèles 9L

Modèle de gliosarcome 9L	Nombre de cellules injectées par rat	Volume d'injection (µL)				Estimation de l'efficacité
		IV	IC	CED	Stéréotaxie	
Sous-cutané	$1.5x10^6$	400	---	---	---	Volume et masse tumoraux
Intracérébral	10^3	400	400	60	10	Temps de survie

IV: injection intraveineuse; IC: injection intra-carotidienne; CED: convection-enhanced delivery

2. Influence des propriétés intrinsèques des suspensions de LNC

Suite aux effets neurologiques secondaires observés dans certains cas lors d'une précédente thèse [9], nous avons mis en place des études de toxicité sur rats sains. 60µl de LNC-FcdiOH à 6.5 mg/g, de LNC blanches ou de sérum physiologique ont été injectés, puis, les rats ont été suivis par imagerie à résonance magnétique (IRM) à J14, J42, J70.

Comme décrit dans la publication n°2, une zone hyper-intense persistante est observée au niveau du côté injecté, ainsi qu'une décroissance des pics des marqueurs de viabilité neuronale. Cette zone apparait aussi bien lorsque le FcdiOH est encapsulé (publication n°2) que lorsque les rats reçoivent des LNC blanches (fait cité dans la publication mais voir Figure 2 non publiée).

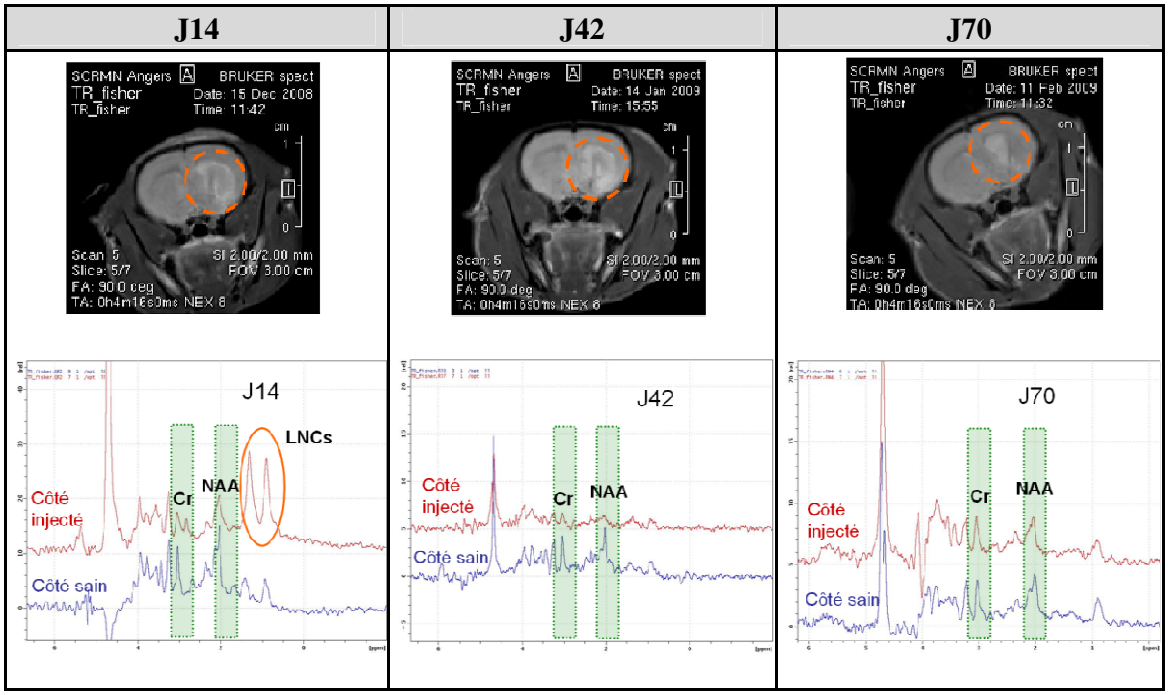


Figure 2 : IRM et RMN des rats ayant reçu des LNC blanches concentrées non-ajustées

Par rapport aux différentes préparations possibles (revue n°1), deux paramètres caractérisent nos formulations de LNC blanches : une forte concentration (réduction du volume d'eau de trempe par rapport à une formulation habituelle) et une absence de dialyse (afin de ne pas diluer la préparation).

La Figure 3 nous montre que la dialyse n'a pas d'influence : la zone hyper-intense est observée dès que les LNC sont concentrées. Par ailleurs, le pH et l'osmolarité ont été mesurés (Tableau 2). Il s'avère que toutes les suspensions de LNC ont un pH légèrement acide et que les formulations concentrées sont fortement hyperosmolaires. L'encapsulation du FcdiOH n'a que très peu d'effet sur les 2 paramètres.

Suite à l'ajustement de l'osmolarité (passage sur colonne PD10) et du pH aux valeurs physiologiques (300 mmol/kg et 7.4 respectivement), l'imagerie et la spectroscopie réalisées chez les rats sains ayant reçu ces suspensions concentrées montrent une zone hyper-intense très limitée sur l'hémisphère affecté et il n'y a plus de différence au niveau de l'intensité des signaux de NAA et Cr (publication n°2).

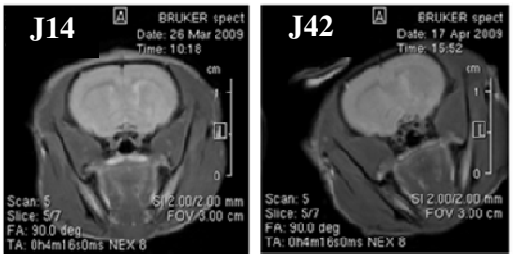
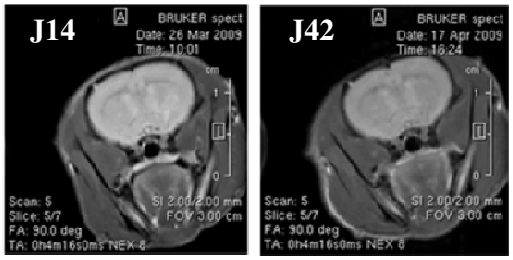
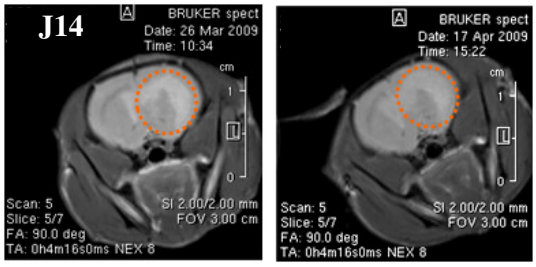
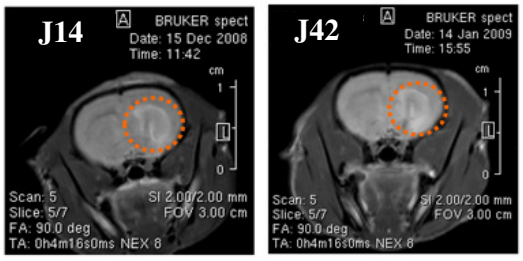
LNC classiques dialysées	LNC classiques non dialysées
	
LNC concentrées dialysées	**LNC concentrées non dialysées**
	

Figure 3 : IRM pondérées T2 des rats ayant reçu différents types de formulation de LNC blanches

Tableau 2 : Paramètres physiques des différents types de formulation de LNC

Formulations		Osmolarité		pH
		Avant colonne PD10	Après colonne PD10	
Concentrées	LNC-FcdiOH	784.00 ± 4.36	179.67 ± 2.08	5.32 ± 0.03
	LNC blanches	780.25 ± 7.80	173.00 ± 4.36	5.39 ± 0.03
Classiques	LNC blanches	227.50 ± 2.12	---	5.63 ± 0.02

La suspension des LNC-FcdiOH 6.5mg/g ajustée a été ensuite injectée par CED chez les rats porteurs de gliosarcome 9L intracérébral pour évaluer leur efficacité. Ce traitement a permis d'augmenter significativement la survie des rats malades par rapport celle du groupe témoin non-traité (28.5 jours par rapport à 25 jours) (Figure 4). Bien que cette amélioration de survie passant de 27 à 28.5 jours reste encore modeste, elle représente cependant un net progrès par rapport aux résultats obtenus jusqu'alors (pour rappel : 23 jours - thèse d'Emilie Allard [9]).

Pour des raisons économiques et éthiques, l'injection des LNC blanches ajustées n'a pas été réalisée. En effet, le traitement par les formulations non ajustées ne donne aucune différence significative avec le groupe témoin non traité (aucun effet positif, aucune toxicité effective) (publication n°1).

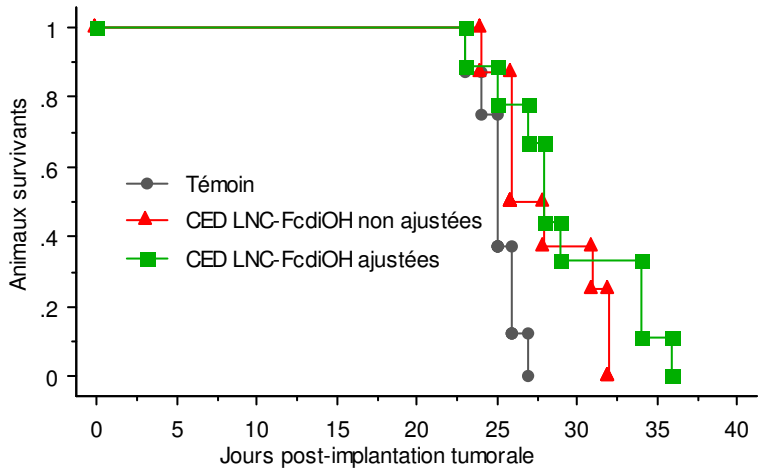


Figure 4 : Courbes de Kaplan-Meier des rats porteurs d'un gliosarcome 9L intracérébral, traités par les LNC-FcdiOH en CED, avec ou sans ajustement de l'osmolarité et du pH

Par ailleurs, en ce qui concerne les injections intravasculaires, la co-administration d'une solution hyperosmolaire telle que le mannitol (20-25% w/v, correspondant à une osmolarité de 1098 à 1372 mmol/kg [10-12]) par voie intra-carotidienne est recommandée pour améliorer la délivrance des principes actifs dans le cerveau [13]. En effet, cela entraîne la vasodilatation et la contraction des cellules endothéliales, résultant en une augmentation de la diffusivité et du débit par ouverture transitoire des jonctions serrées de la BHE [14-15]. C'est la raison pour laquelle les suspensions hyperosmolaires de LNC ont été utilisées telles quelles pour les injections intra-carotidiennes et intraveineuses réalisées par la suite dans ce travail.

3. Influence de la dose de FcdiOH

Afin d'évaluer l'influence de la dose de FcdiOH administrée, une gamme de concentration a été réalisée par CED chez les rats porteurs d'un gliosarcome 9L intracérébral. Comme attendu, l'effet cytostatique du FcdiOH est proportionnel à la dose puisque les médianes de survie des rats traités augmentent de 24 à 27 puis à 31 jours pour des doses de 0.005 (10µL LNC-FcdiOH 0.5mg/g), 0.36 (60µL LNC-FcdiOH 6.5mg/g) et 2.4mg/rat (60µL FcdiOH dans le Labrafac à 40 mg/g) (publication n°1).

Malheureusement, même si le Labrafac® permet d'augmenter les doses, son injection directe semble toxique pour le parenchyme cérébral. En effet, les rats traités par le Labrafac® seul montrent des signes de toxicité (léthargie, perte de poids…) et présentent une survie significativement plus courte que celle des rats témoins non traités (21.5 par rapport à 25 jours). De plus, l'IRM effectué chez un rat long survivant obtenu avec la solution FcdiOH/Labrafac® montre une rétention, jusqu'à J100, des triglycérides mise en évidence par des zones hyper-intenses sur les images de pondération T1 [9]. Cela témoigne d'une non-biocompatibilité de ce produit et de la nécessité d'encapsulation du FcdiOH dans les LNC avant leur administration.

Dans ce contexte montrant qu'une dose élevée peut être efficace sur la survie, nous avons cherché à augmenter cette dose en jouant sur :

- la fréquence d'administration (non-publié)
- le taux de charge en principe actif au cœur des LNC (non-publié)
- le volume d'injection (publication n°2).

Tout d'abord, des multi-injections ont été envisagées. Les rats ont subi deux injections de LNC-FcdiOH 6.5mg/g par CED, 6 et 12 jours après implantation tumorale. Cependant, l'augmentation de la dose n'améliore pas la survie des rats et la médiane demeure à 27 jours. De plus, une perte de poids des rats traités pouvant être due au protocole invasif de cette double CED (double intervention, double anesthésie, durée de l'opération allongée, …) a été également observée (Figure 5).

Par ailleurs, un protocole moins lourd qui s'est révélé très efficace pour la radiothérapie interne avec des LNC chargées en complexe de Rhénium-188 [$^{188}Re(S_3CPh)_2(S_2CPh)$ = ^{188}Re-SSS] [16], a aussi été appliqué : stéréotaxie à J6 puis CED à J12. Malheureusement, ce protocole n'est pas applicable dans le cas des LNC-FcdiOH puisque la médiane de survie des rats reste de 24 jours (Figure 5). Il semble que la dose injectée par stéréotaxie à J6 (10µL LNC, 0.06 mg FcdiOH/rat) soit trop faible pour entraîner un effet thérapeutique et que la CED à J12 intervienne trop tard pour éradiquer une tumeur qui s'accroît très rapidement (volume de 17±10µl à J14 passant à 167±19µl à J24) [17].

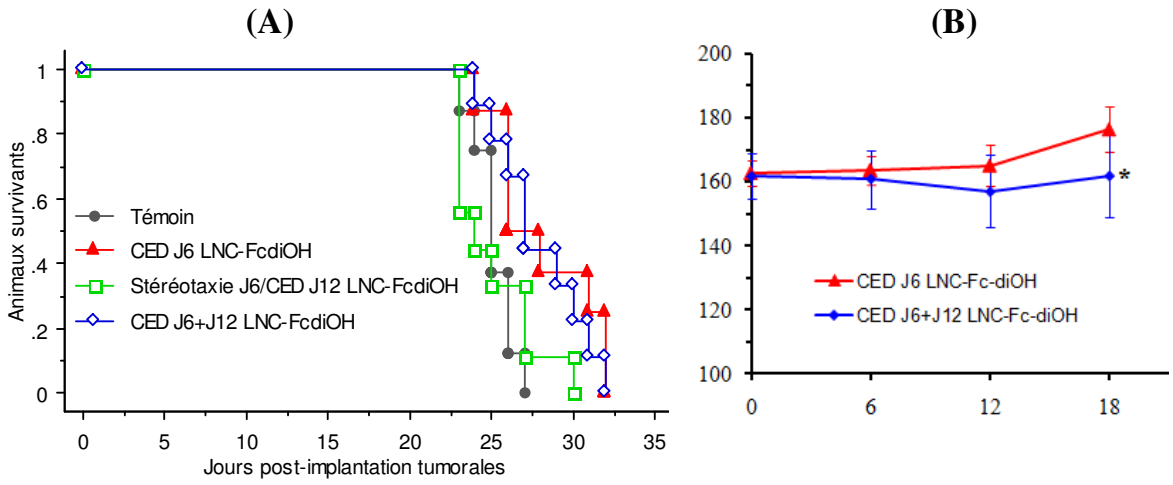


Figure 5 : Courbes de Kaplan-Meier (A) et des poids moyens (B) des rats porteurs d'un gliosarcome 9L intracérébral, traités par une injection unique à J6 ou deux injections à J6 et J12 en stéréotaxie/CED de LNC-FcdiOH

Toujours dans l'optique d'augmenter la dose infusée, le taux de charge en FcdiOH dans le Labrafac a été augmenté jusqu'à 110 mg/g (au lieu de 40 mg/g). A cette concentration, le FcdiOH ne se dissout pas, même en passant la préparation aux ultra-sons. Cependant, grâce

aux 3 cycles de température effectués entre 85 et 60^0C lors de la formulation des nanocapsules, le FcdiOH se solubilise et s'encapsule dans les LNC pour aboutir à des LNC-FcdiOH à 18.5mg/g (au lieu de 6.5mg/g). Cette suspension a été injectée par CED chez les rats expérimentaux à J6. Malheureusement, cette dose apparait toxique puisque 4 rats sur 6 meurent 2 jours après infusion du traitement et 2 autres à J20 et J22, donc 3 à 5 jours plus tôt que les rats témoins non traités (médiane de 25 jours). Une hypothèse peut être avancée afin d'expliquer ce résultat négatif. En effet, il est connu (*in vitro)* que les LNC améliorent l'internalisation intracellulaire des principes actifs [18]. Si cela est également le cas *in vivo*, une surexposition des cellules tumorales ainsi que des cellules saines au principe actif encapsulé dans les LNC, pourrait entraîner une certaine toxicité. En effet, alors qu'une cytostaticité est attendue pour inhiber la progression de la tumeur, une cytotoxicité par surdosage peut entraîner la mort des cellules par nécrose. La nécrose est une forme pro-inflammatoire de la mort cellulaire accompagnée par la libération de protéines et acides nucléiques spécifiques dans l'espace extracellulaire [19]. Ces médiateurs sont capables de favoriser la mise en route d'une réponse inflammatoire et d'entrainer des effets négatifs sur les cellules saines de l'entourage [20-21].

Enfin, l'augmentation du volume d'injection a été étudiée. Néanmoins, l'administration directe dans le cerveau par CED est limitée par le volume d'infusion, pour des raisons de tolérance clinique. Dans une approche moins invasive et après modification de la surface des LNC-FcdiOH (ajout de longues chaines de PEG), l'injection intra-carotidienne a alors été envisagée comme une voie alternative pour la délivrance des LNC-FcdiOH. Effectivement, cette voie d'administration permet d'augmenter le volume d'injection à 400µL, donnant lieu à une augmentation de la dose en FcdiOH jusqu'à 2.4 mg/rat. Cela a permis d'obtenir une amélioration significative de la médiane de survie des rats traités par rapport au groupe témoin (30 jours par rapport à 25, correspondant à une augmentation importante de 20%) (publication n°2). De manière intéressante, la survie des rats dans ce cas rejoint celle du groupe des rats traités par le FcdiOH solubilisé dans le Labrafac à la même dose (P = 0.3842, Logrank test).

Ces résultats contribuent à confirmer l'influence de la dose sur l'efficacité thérapeutique du FcdiOH. Ils démontrent par ailleurs l'intérêt de ces nanovecteurs dans le cadre d'une chimiothérapie avec le FcdiOH et, plus largement, pour la délivrance de principes actifs dans le cerveau.

4. Protection stérique : avantages et inconvénients

Bien que la surface des LNC classiques (de première génération) soit recouverte par des PEG 660 à haute densité, elles sont rapidement éliminées de la circulation sanguine, avec une demi-vie plasmatique d'environ 21-22 min chez le rat [22-23]. Cela reste insuffisant pour permettre une accumulation dans les tissus tumoraux après injection intraveineuse par effet EPR (enhanced-permeability and retention) [24]. L'élaboration des LNC de deuxième génération a donc été nécessaire (revues n°1 et n°2) pour s'inscrire dans une stratégie de ciblage passif en thérapie cancéreuse. Celle-ci est basée principalement sur la conception d'une protection stérique conférée par de longues chaines de PEG post-insérées à la surface des LNC [24-25]. Ainsi, les LNC recouvertes par des chaines de DSPE-mPEG2000 ont été évaluées en tant que nanovecteurs de FcdiOH administrés par les différentes voies étudiées.

4.1. Injection intraveineuse

Après recouvrement par le DSPE-mPEG2000, les LNC pégylées présentent une demi-vie 4 fois plus longue et une aire sous la courbe (area under the plasma concentration time – AUC) 1.65 fois plus étendue que celle des LNC classiques chez le rat sain après injection intraveineuse. Tout d'abord, l'effet antitumoral du FcdiOH encapsulé dans les LNC recouvertes ou non avec des DSPE-mPEG2000 a été évalué sur un modèle de gliosarcome 9L sous-cutané. Le traitement avec les LNC-FcdiOH a diminué la croissance tumorale par rapport à l'injection de sérum physiologique ou à celle des LNC blanches et, de manière intéressante, l'injection de DSPE-mPEG2000-LNC-FcdiOH a permis d'éradiquer la tumeur. En comparaison avec l'étude précédente d'Emilie Allard effectuée sur le même modèle de gliosarcome sous-cutané dans laquelle les LNC-FcdiOH avaient été administrées par injection intratumorale à J6 [26], le passage par la vascularisation et donc le ciblage passif semblent plus efficaces (publication n°3).

Afin de mesurer la puissance de notre anticancéreux, nous avons voulu comparer l'efficacité des LNC-FcdiOH avec des LNC chargées en Paclitaxel (PTX), bien connu pour son efficacité sur un large spectre de cancers (sein, ovaire, poumon à petite cellule et non-petite cellule, et sarcome de Kaposi associé au SIDA) [27] (résultats non publiés).

Les LNC-PTX ont été préparées suivant le protocole appliqué pour les LNC-FcdiOH. Brièvement, le PTX a été mélangé au Labrafac® à une concentration de 40 mg/g sous l'action des ultra-sons pendant 1h30 avant l'ajout des autres ingrédients (Solutol, Lipoid, NaCl et eau). Les 3 cycles de température entre 60 et 85^0C ont été effectués et suivis de l'ajout d'eau froide pour figer le système et aboutir aux LNC-PTX avec une concentration théorique de 6.5 mg/g. Cependant, le dosage par HPLC [28] après passage sur un filtre hydrophile de 0.2µm a mis en évidence un taux de charge de 2 mg/g, correspondant à un faible rendement d'encapsulation de 30% qui sera discuté plus loin. Les DSPE-mPEG2000 ont été ensuite incorporés à la surface des LNC-PTX par la technique de post-insertion à 60^0C pendant 2h.

Les LNC-PTX et DSPE-mPEG2000-LNC-PTX ont été injectées par voie intraveineuse chez les rats porteurs d'un gliosarcome 9L sous-cutané. Les LNC-PTX ont donné lieu à une diminution du volume et de la masse de la tumeur, de manière à priori comparable aux LNC-FcdiOH (Figure 6). Les écart-types montrent cependant que l'effet des LNC-PTX n'est pas significativement différent de celui obtenu avec les LNC blanches. Le traitement avec les DSPE-mPEG2000-LNC-PTX a freiné la croissance des tumeurs de manière identique aux LNC-PTX (pas de différence significative), sans les éradiquer.

L'échec du traitement avec les DSPE-mPEG2000-LNC-PTX pourrait être lié à un problème de formulation. Effectivement, un relargage rapide du PTX hors des LNC a été observé sous forme d'un précipité au cours de la conservation des LNC-PTX à 4^0C. De plus, Hureaux *et al.* a récemment montré l'influence de la température de stockage sur la stabilité de ces LNC, révélant une augmentation de la taille et de l'index de polydispersité avec l'augmentation de la température et recommandant de les congeler immédiatement après préparation puis de les stocker dans l'azote liquide [29]. Dans ces conditions, le faible rendement d'encapsulation (30%) peut s'expliquer par une fuite du PTX, et il est possible que cette fuite s'accentue lors de la post-insertion des DSPE-mPEG2000 qui s'effectue à 60°C.

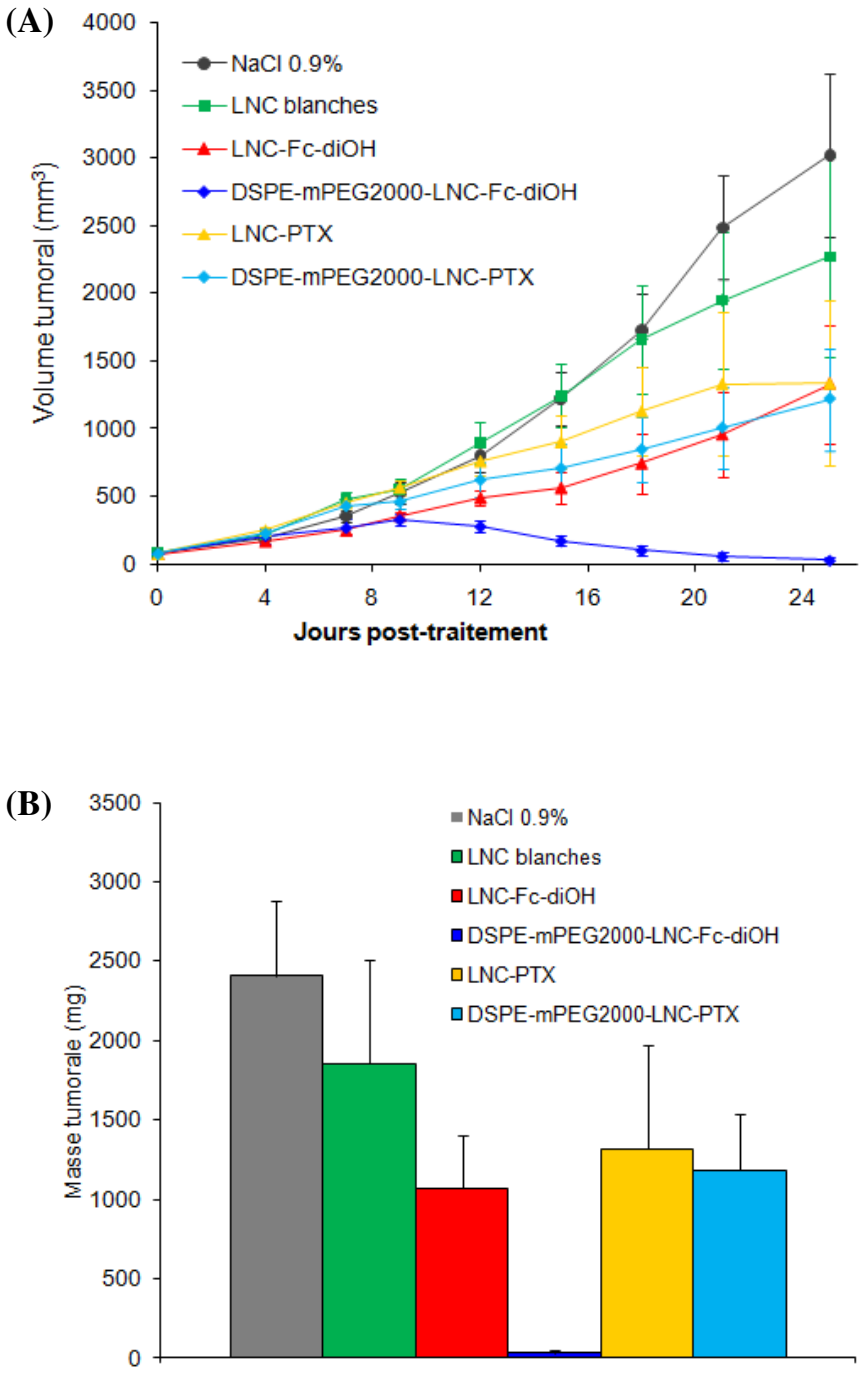


Figure 6 : Suivi du volume tumoral (A) et de la masse tumorale (B) pendant 25 jours sur des rats porteurs de gliosarcome 9L sous-cutané, traités avec les LNC contenant soit du FcdiOH soit du paclitaxel (PTX) par voie intraveineuse

En résumé, les résultats obtenus avec les nanovecteurs chargés en FcdiOH sur le modèle ectopique de gliosarcome sont très prometteurs. En effet, un grand nombre d'études dans la littérature concernant le traitement systémique sont généralement réalisées à partir d'injections répétées [30-33] ou bien d'un traitement combiné, tel que cisplatine et thalidomide [34], afin d'atteindre une efficacité significative. Dans notre cas, une seule injection de DSPE-mPEG2000-LNC-FcdiOH a fait disparaitre la tumeur en 25 jours. Ces résultats démontrent, d'une part, l'accumulation passive des nanovecteurs dans les tumeurs, entrainant un apport important de principe actif sur le site ciblé, et, d'autre part, l'efficacité antitumorale *in vivo* du FcdiOH. Cela ouvre également de nombreuses perspectives concernant l'utilisation des LNC-FcdiOH dans d'autres modèles cancéreux, notamment des modèles orthotopiques hautement vascularisés qui répondraient à ce principe actif.

Puis, ces résultats positifs nous ont conduits à évaluer nos nanovecteurs dans un modèle cérébral orthotopique. Dans cette optique, les DSPE-mPEG2000-LNC-FcdiOH et LNC-FcdiOH ont été injectées par voie intraveineuse à des rats 6 jours après implantation d'un gliosarcome 9L au niveau du cerveau. Les résultats de l'étude de survie montrent que le traitement par les LNC-FcdiOH classiques ou pégylées augmente légèrement, mais significativement du point de vue statistique, la médiane de survie des rats par rapport à celle du groupe témoin (27.5 et 28 jours, respectivement par rapport à 25 jours) (publication n°3). Cette augmentation reste encore modeste mais est à remarquer car elle fait suite à une injection intraveineuse unique donnant un effet sur la tumeur pourtant localisée dans le cerveau, et ce, malgré la présence de la BHE (tumeur très petite à J6) [17].

Enfin, la capacité de délivrance de principes actifs à travers la BHE par des nanoparticules protégées par le Tween® 80 a été mise en évidence dans de nombreuses études de l'équipe de Kreuter [35-38]. Le Tween® 80 ou le Polysorbate 80, est un polyester de sorbitane de PEG et d'acide oléique (Figure 7). Dans le cas des nanoparticules de poly(butyl cyanoacrylate) (PBCA) incubées avec du Tween 80, le mécanisme proposé est médié par les récepteurs aux lipoprotéines de basse densité, grâce à l'adsorption sélective des apolipoprotéines A-1 et/ou E à la surface des nanoparticules. Une endocytose permet alors un passage de ces vecteurs à travers la BHE [39].

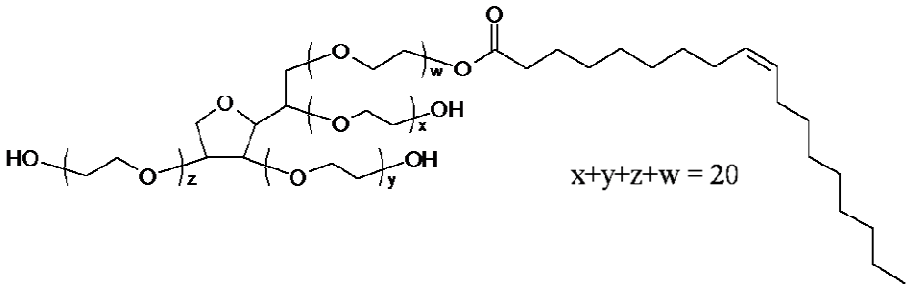


Figure 7 : Structure chimique du Tween® 80

Dans cette optique, les LNC-FcdiOH ont été incubées avec 1% (w/v) de Tween® 80 pendant 30min et ensuite injectées dans un modèle orthotopique (résultats non publiés). Ce traitement a permis d'augmenter légèrement la survie des animaux avec une médiane de 28 jours (Figure 8), ce qui est similaire à ce qui est obtenu avec les DSPE-mPEG2000-LNC-FcdiOH et nous conforte dans l'utilisation de ce recouvrement.

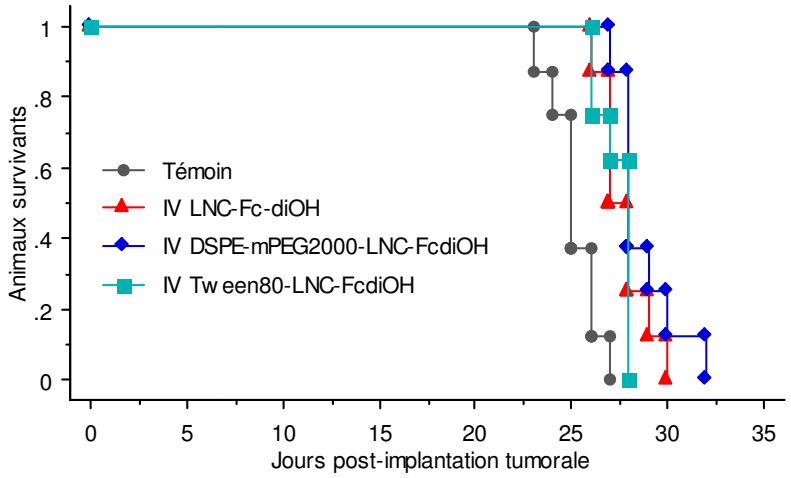


Figure 8 : Courbes de Kaplan-Meier des rats porteurs d'un gliosarcome 9L intracérébral, traités avec différents types de LNC-FcdiOH par voie intraveineuse (IV)

4.2. Injection intra-carotidienne

L'administration des traitements par cette voie consiste en une injection locorégionale dans l'artère carotidienne permettant d'augmenter le volume d'injection par rapport à la CED (400µL au lieu de 60µL). Le marquage des LNC par un fluorochrome hydrophobe (1,10-dioctadécyl-3,3,30,30-tétraméthylindocarbocyanine perchlorate, DiI) nous a permis d'estimer la distribution de différents types de LNC dans le cerveau des rats. L'observation sous microscope à fluorescence des coupes coronales a mis en évidence une augmentation de l'accumulation de divers types de LNC dans le cerveau 24h après une injection intra-carotidienne par rapport une injection intraveineuse (Figure 9).

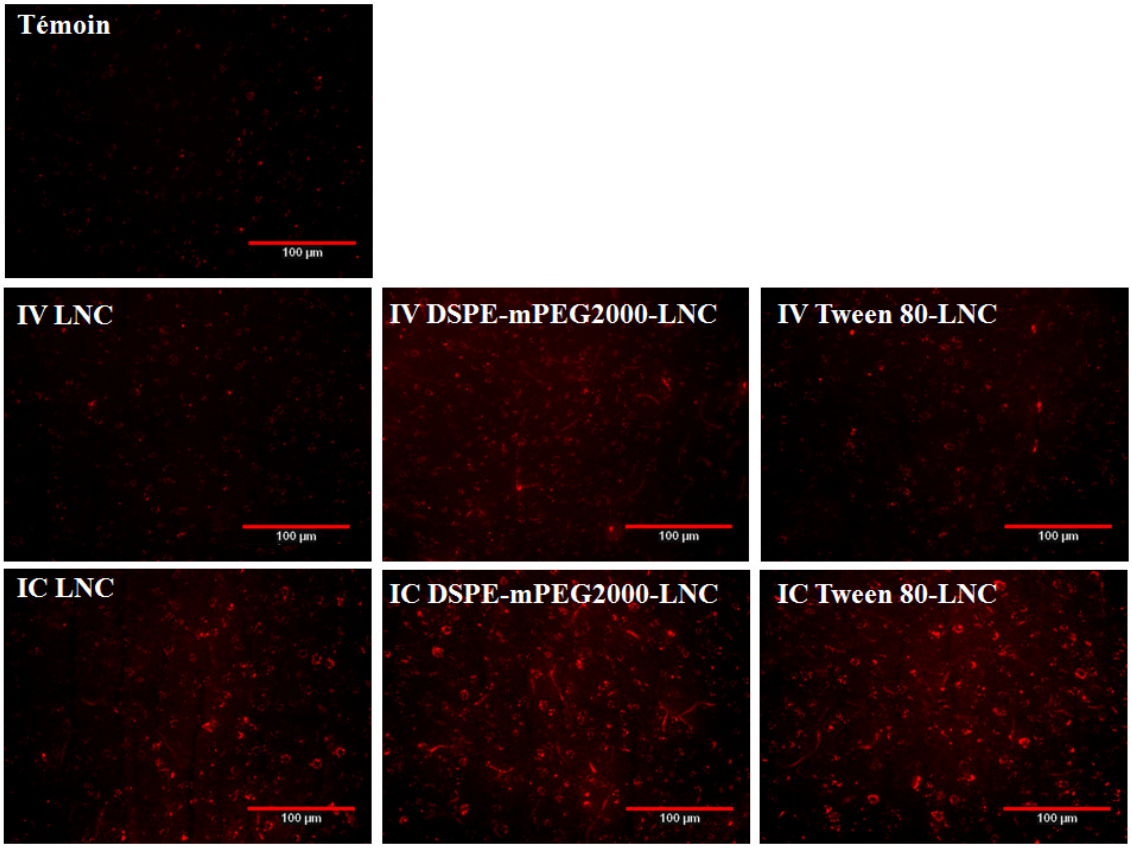


Figure 9: Images fluorescentes des coupes de cerveau des rats porteurs d'un gliosarcome 9L intracérébral, ayant reçu des LNC-DiI, DSPE-mPEG2000-LNC-DiI ou Tween 80-LNC-DiI par voie intraveineuse (IV) ou intra-carotidienne (IC)

Si l'on compare les DSPE-mPEG2000-LNC aux Tween 80-LNC, on peut constater que l'accumulation cérébrale est plus importante pour les DSPE-mPEG2000-LNC, ce qui est mis en évidence par l'apparition de faisceaux lumineux sans doute représentatifs de capillaires marqués. Ces observations sont confirmées de façon semi-quantitative par une mesure des surfaces fluorescentes effectuée sur les mêmes coupes de cerveau, montrant également des différences significatives par rapport aux LNC non recouvertes (Figure 10). Par ailleurs, ces résultats montrent nettement l'intérêt de la voie d'administration intra-carotidienne par rapport à la voie intraveineuse.

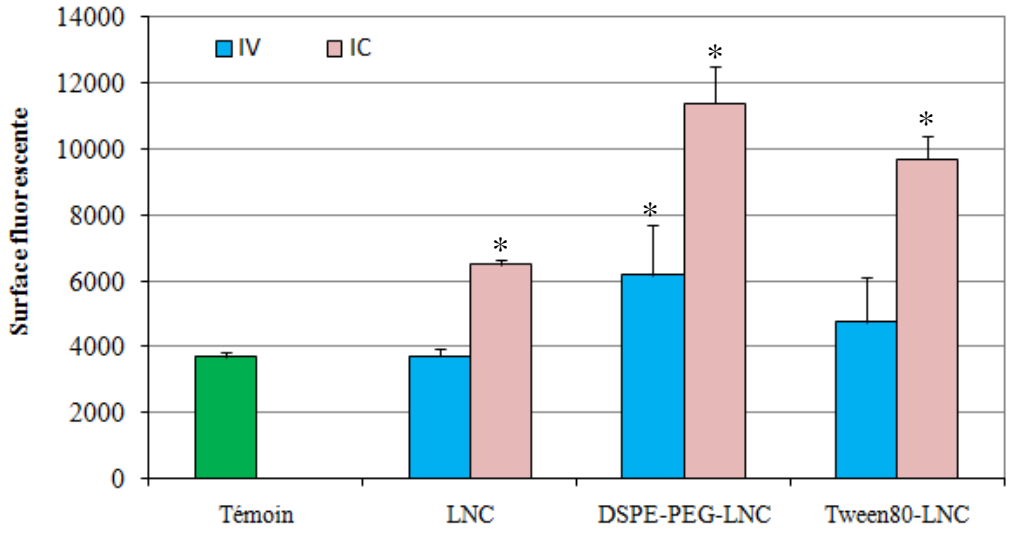


Figure 10: Quantification de la présence des LNC marquées au DiI sur des coupes de cerveau par mesure des surfaces fluorescentes ((*) indique $P < 0.05$ par rapport au témoin)

L'encapsulation du FcdiOH dans ces différents vecteurs et l'évaluation de leur efficacité sur un modèle de gliosarcome intracérébral nous a également permis de confirmer les avantages apportés par l'administration intra-carotidienne des DSPE-mPEG2000-LNC. En effet, les groupes traités avec les LNC-FcdiOH et les Tween 80-LNC-FcdiOH possèdent des médianes de survie 27 et 27.5 jours respectivement alors que le traitement par les DSPE-mPEG2000-LNC donne une médiane de 30 jours (Figure 11). L'utilisation du Tween® 80 n'améliore donc pas l'efficacité antitumorale des LNC-FcdiOH par voie intra-carotidienne. De plus,

certains inconvénients du Tween® 80 sont évoqués dans la littérature, tels que la modulation de la perméabilité non-spécifique de la BHE observés *in vitro* et *in vivo* ainsi que le problème d'efficacité avérée par rapport à d'autres systèmes pégylés (comme nous l'avons observé nous-mêmes en injection intraveineuse) [40-41]. Par conséquent, nous n'avons pas poursuivi l'optimisation de la chimiothérapie à l'aide du Tween® 80.

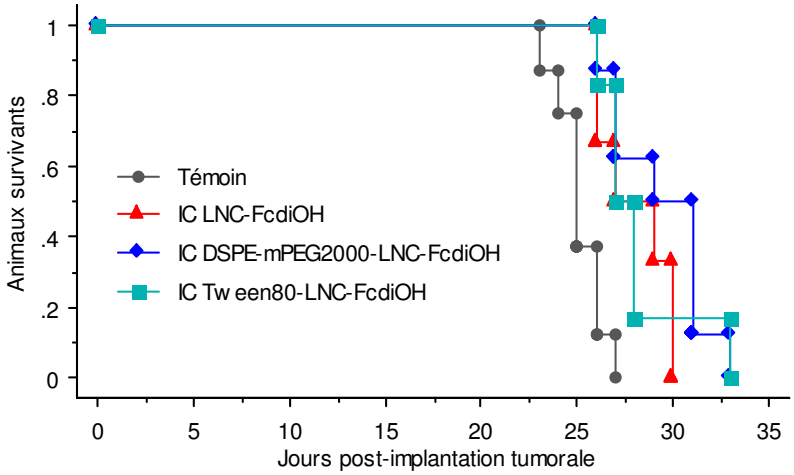


Figure 11 : Courbes de Kaplan-Meier des rats porteurs d'un gliosarcome 9L intracérébral, traités avec différents types de LNC-FcdiOH par voie intra-carotidienne (IC)

4.3. Convection-enhanced delivery (CED)

A partir des résultats intéressants obtenus par traitement intraveineux et intra-carotidien avec les DSPE-mPEG2000-LNC-FcdiOH, nous avons injecté ces nanovecteurs par CED dans un modèle orthotopique à J6 sachant que la suspension a été ajustée au niveau de son osmolarité et de son pH pour s'adapter aux valeurs physiologiques. Malheureusement, la survie des rats n'est pas prolongée par ce traitement car leur médiane de survie est alors de 24 jours par rapport à 25 jours pour les témoins. Cela met en évidence un effet négatif dû au recouvrement des nanocapsules par de longues chaines de PEG, lorsque ces nanovecteurs sont directement implantés sur le site tumoral. De nombreuses études de la littérature ont déjà montré le problème de faible biodisponibilité des nanoparticules pégylées, le PEG empêchant l'interaction des nanovecteurs avec les cellules tumorales ainsi que la libération des principes actifs [42-45]. De plus, le PEG, lui-même, pourrait entraîner potentiellement certaines

toxicités telle que le déclenchement d'une réponse immunitaire ou des réactions allergiques (revue n°2). A ce niveau, le développement de nanovecteurs de troisième génération permettant de favoriser le ciblage spécifique et l'internalisation au sein des cellules tumorales devient séduisant.

5. Incorporation d'anticorps ou de peptides

Au sein du laboratoire, des immunonanocapsules ont été développées récemment par greffage covalent à la surface des LNC d'anticorps monoclonaux OX26 dirigés contre le récepteur à la transferrine de rat [4]. Ce dernier est surexprimé sur les cellules endothéliales cérébrales de la BHE [46]. Les études précédentes ont montré une fixation spécifique *in vitro* de ces immunonanocapsules sur les cellules surexprimant le récepteur à la transferrine (hybridomes Y3.AG.1.2.3. et cellules endothéliales cérébrales de rat), entraînant une légère accumulation dans le cerveau de rats sains après injection intraveineuse [47]. Par ailleurs, les récepteurs à la transferrine sont également surexprimés à la surface des cellules à forte activité mitotique comme les cellules de gliome [48], ce qui pourrait permettre de favoriser l'internalisation des nanovecteurs dans les cellules tumorales et entraîner une amélioration de l'effet thérapeutique des principes actifs encapsulés.

Ces immunonanocapsules ont été injectés par voie intra-carotidienne ou par CED (les deux voies les plus prometteuses) chez les rats porteurs de gliosarcome intracérébral à J6 (résultats non publiés). Le traitement intra-carotidien avec les OX26-LNC-FcdiOH semble inefficace puisque la survie moyenne ne change pas par rapport à celle du groupe témoin (25 jours). De plus, les rats traités par CED présentent une médiane de survie plus courte que celle des témoins (22 jours au lieu de 25) (Tableau 3 et Figure 12). Ces résultats négatifs pourraient être reliés à la concentration insuffisante testée (3.4 mg/g au lieu de 6.5 mg/g pour la formulation habituelle), conséquence de certaines dilutions réalisées au cours de la préparation. Par ailleurs, même si l'accumulation des immunocapsules dans le cerveau a été prouvée après injection [47], il est possible que la plus grande partie reste dans les cellules endothéliales capillaires et non pas dans le compartiment parenchymateux [49], suite à une forte affinité des anticorps avec leur récepteur. Enfin, l'échec du traitement avec les OX26-LNC-FcdiOH pourrait être dû à la chimie de préparation de ces nanovecteurs. En effet, lors du couplage covalent de l'OX26 à la surface des LNC par le biais de la fonction maléïmide,

un risque potentiel de formation d'hydrazones *in situ* résultant de la réaction d'un des composés mis en excès (PDPH) avec des intermédiaires métaboliques, du sucre réduit présent dans les cellules ou des lysats cellulaires pourrait entrainer un problème de cytotoxicité et d'inhibition des enzymes *in vivo* [50].

Tableau 3 : Tableau récapitulatif des données de survie des rats porteurs de gliosarcome 9L intracérébral, traités avec les OX26-LNC-FcdiOH et les peptide-LNC-FcdiOH par CED ou injection intra-carotidienne (IC)

Voie d'admin.	Traitement	Dose de FcdiOH (mg/rat)	n	Temps de survie (jours)			% ATS	
				Min-Max	Médiane	Moyenne ± DS	Médiane	Moyenne
IC	Peptide-LNC (5.5 mg/g)	2.2	6	24-44	28	29.50 ± 7.45	12	17.41
	OX26-LNC (3.4 mg/g)	1.36	6	23-28	24.5	25.17 ± 1.94	---	0.17
CED	Peptide-LNC (5.5 mg/g)	0.33	3	20-22	21	21.00 ± 1.00	---	---
	OX26-LNC (3.4 mg/g)	0.20	5	20-25	22	22.00 ± 1.87	---	---
	Témoins	---	8	23-27	25	25.13 ± 1.25	---	---

Admin. : administration ; % ATS : pourcentage d'accroissement du temps de survie ; DS : déviation standard

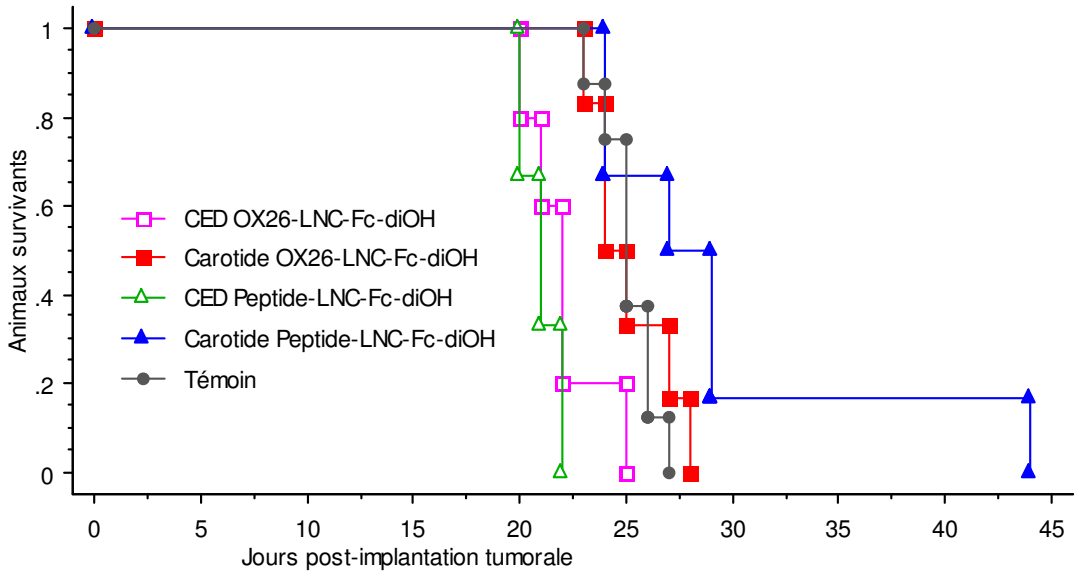


Figure 12 : Courbes de Kaplan-Meier des rats porteurs d'un gliosarcome 9L intracérébral, traités avec les OX26-LNC-FcdiOH et les peptide-LNC-FcdiOH par CED et par voie intra-carotidienne

En parallèle, des peptides contenant des séquences d'acides aminés des protéines de neurofilament (NFL) qui interagissent spécialement avec les sites de liaison de la tubuline (tubulin-binding sites TBS), synthétisés et brevetés par l'équipe du Dr. Joël Eyer (Laboratoire Neurobiologie et Transgénèse Inserm, EA3143, Angers) ont été testés (publication n°4). Le peptide de NFL-TBS a montré une bonne internalisation dans les cellules de gliome en culture et également sur les modèles animaux [51-52]. Les résultats du test MTS sur la viabilité *in vitro* des cellules 9L ont montré que l'incorporation des peptides à la surface des LNC permet d'améliorer la cytostaticité des LNC-FcdiOH. Cependant, l'administration des peptide-LNC-FcdiOH par CED a entraîné 50% de mortalité (2 jours après le traitement) et présente une médiane de survie de 21 jours, qui pourrait être la conséquence d'un problème de surdosage et/ou de toxicité/inflammation se déclenchant dans le cerveau des animaux. En revanche, l'administration intra-carotidienne des peptide-LNC-FcdiOH a prolongé le temps de survie des animaux avec un rat survivant jusqu'à 44 jours (publication n°4). Un tel temps de survie n'a été obtenu qu'exceptionnellement, malgré la diversité des traitements testés dans ce travail, et est à prendre en considération. Cependant, le nombre de rats testés reste encore faible et la répétition de ce traitement sur un grand nombre d'animaux devra être effectuée pour confirmer ce résultat.

6. Conclusion

En résumé, l'ensemble des résultats exposés dans la figure 13 montre l'intérêt de l'administration locale par CED et locorégionale par voie intra-carotidienne dans notre étude. Les LNC-FcdiOH ajustées mais non fonctionnalisées semblent les plus efficaces pour une administration locale par CED. En revanche, pour une administration intra-carotidienne, les stratégies de ciblage passif (DSPE-mPEG2000) ou actif (peptide NFL-TBS) ouvrent des perspectives prometteuses.

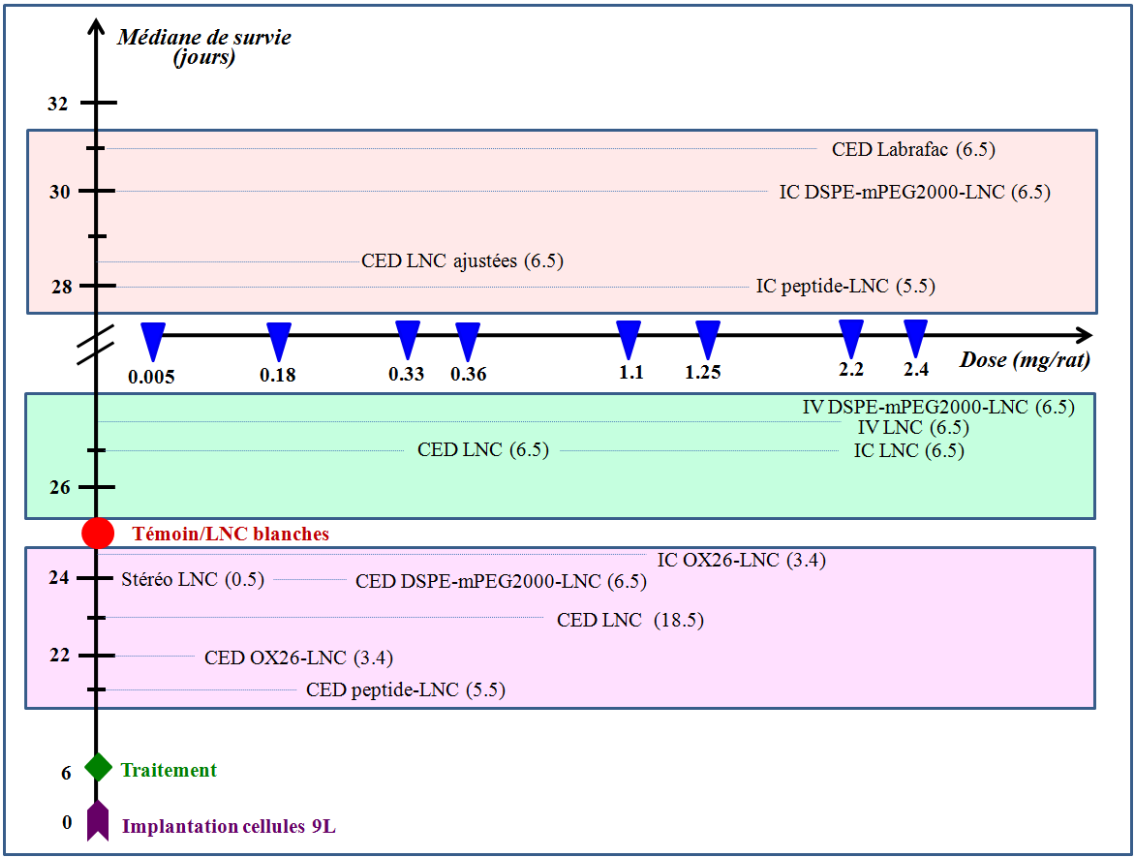


Figure 13 : Médianes de survie des rats porteurs d'un gliosarcome 9L intracérébral, traités avec différents types de LNC-FcdiOH ou FcdiOH/Labrafac® à différents taux de charge (concentration en mg/g entre parenthèses), administrés par stéréotaxie (stéréo), convection-enhanced delivery (CED), injection intra-carotidienne (IC) et injection intraveineuse (IV)

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CONCLUSION & PERSPECTIVES

L'administration locale par CED (60µL) des LNC-FcdiOH dont l'osmolarité et le pH ont été ajustés aux valeurs physiologiques a permis de préserver l'environnement cérébral des rats sains tout en augmentant significativement la survie des rats malades par rapport à celle du groupe témoin non traité (médiane de 28.5 jours et de 25 jours, respectivement). Afin d'augmenter le volume d'injection (400µL), 2 voies d'administration intra-vasculaire ont été envisagées : l'injection intraveineuse et l'injection intra-carotidienne. Dans le cadre d'un ciblage passif, les LNC ont été recouvertes par de longues chaines de PEG (DSPE-mPEG2000) afin de leur conférer des propriétés furtives. Les LNC pégylées ont présenté un meilleur temps de circulation sanguine que celui des LNC classiques. Cela a permis d'aboutir à l'éradication de la tumeur 9L sous-cutanée après une injection intraveineuse des DSPE-mPEG2000-LNC-FcdiOH. En parallèle, l'injection intra-carotidienne représente une voie d'administration prometteuse pour la délivrance de principes actifs dans le cerveau. De façon encourageante, le traitement intra-carotidien à l'aide des DSPE-mPEG2000-LNC-FcdiOH a entraîné une augmentation importante de la médiane de survie des rats porteurs d'un gliosarcome 9L intracérébral (20% par rapport à celle du groupe témoin). Enfin, les résultats préliminaires obtenus dans le cadre d'un ciblage actif par incorporation d'anticorps OX26 ou de peptide, le NFL-TBS, ont montré que seul ce dernier pouvait donner lieu à un effet positif dans les conditions testées (médiane de survie de 28 jours - un survivant à 44 jours).

Les perspectives à venir concernent tout d'abord une étude approfondie de la relation efficacité-toxicité, indispensable au développement futur de nos nanovecteurs. La dose minimale efficace (MED : minimum effective dose) ainsi que la dose maximale tolérée (MTD : maximum tolerated dose) par des animaux après administration des LNC-FcdiOH devront être évaluées. Ces paramètres, très importants pour un agent anticancéreux qui possède une marge thérapeutique étroite [1], sont déjà en cours d'évaluation dans le cadre d'un programme ANR concernant l'étude mécanistique des ferrocifènes (MECAFERROL). Comme observé dans ce travail, l'administration par CED d'une forte concentration de FcdiOH (18.5mg/g) ou des nanovecteurs recouverts de peptide, de concentration moins élevée mais pénétrant probablement mieux dans les cellules tumorales, semble montrer qu'une dose toxique est atteinte (surdosage). Des études plus approfondies sur cette relation efficacité-toxicité, en variant la dose administrée sur un grand nombre d'animaux, devraient permettre d'ouvrir de nouvelles pistes notamment dans le cadre du ciblage actif.

En parallèle, des études de libération *in vitro* du FcdiOH permettant de comparer les différents nanovecteurs devront être réalisées, notamment en milieu aqueux (tampon PBS, pH 7.4) et en milieu LCR (liquide céphalo-rachidien) afin de prédire la libération du principe actif dans les tissus cibles, après injection. Pour ce faire, il faudrait mettre en place une procédure de dosage du FcdiOH par HPLC (chromatographie en phase liquide à haute performance) permettant de détecter le principe actif à faible concentration.

Le traitement par voie intra-carotidienne s'est avéré efficace mais reste à optimiser. En effet, certaines complications sont décrites dans la littérature, principalement des perturbations locales ophtalmiques et neurologiques [2-3], après administration intra-carotidienne d'agents anticancéreux, tels que cisplatine, étoposide, 5-fluorouracil, carmustine/BCNU [4-8]. De plus, l'utilisation de suspensions hyperosmolaires de LNC permettant l'ouverture transitoire de la BHE pourrait entrainer également des effets secondaires [9]. Par conséquent, une étude similaire à celle réalisée après injection en CED par imagerie à résonance magnétique est à effectuer à différents temps après injection. Par ailleurs, la réversibilité et la durée de rupture de la BHE sont des données essentielles qui pourront être déterminées en utilisant un colorant (bleu Evans) comme marqueur de la perméabilité [10].

Par ailleurs, les tumeurs présentent une hypertension interstitielle tandis que la pression du fluide interstitiel est presque nulle dans les tissus normaux [11]. Ainsi, malgré l'effet EPR, le franchissement de la paroi des vaisseaux sanguins et la migration entre les espaces interstitiels tumoraux après extravasation sont négligeables. Ceci empêche l'homogénéité de la distribution des agents thérapeutiques dans les tissus tumoraux. De plus, il est bien connu que le microenvironnement tumoral est caractérisé par des phénomènes d'hypoxie et d'acidose, conséquences métaboliques du déséquilibre entre le développement du réseau vasculaire et la prolifération rapide des cellules tumorales [12]. Dans le cas du FcdiOH, dont le mécanisme d'action proposé est basé sur l'oxydation intramoléculaire par un transfert d'électrons permettant de former des métabolites cytostatiques [13], ces propriétés peuvent inhiber l'activation de cette molécule et potentiellement diminuer son efficacité antitumorale. Dans ce contexte, la co-administration d'un effecteur allostérique d'hémoglobine, par exemple le myo-inositol trispyrophosphate [14], pourrait être à considérer. Ce genre de molécule permet de favoriser le relargage d'oxygène à partir des globules rouges et donc d'améliorer l'oxygénation tumorale.

D'autre part, la lignée cellulaire de gliosarcome de rat 9L représente une lignée intéressante pour des études de gliome malin chez animal. Cependant ce modèle présente une forte immunogénicité, contrairement au gliome humain, entraînant potentiellement une régression spontanée sans intervention thérapeutique. Cette propriété peut être problématique dans l'évaluation de l'effet anticancéreux à long-terme [15]. La migration vers un modèle de gliome F98, faiblement immunogénique et plus proche du gliome humain, serait intéressante. L'utilisation de modèles humains sur souris nude est également envisageable.

Finalement, dans une optique conforme aux critères éthiques concernant les expérimentations sur les animaux, il serait utile de mettre en place un protocole d'évaluation de l'efficacité thérapeutique autre que la survie des animaux. Ceux-ci pourraient être sacrifiés un jour donné après implantation des cellules tumorales, minimisant les souffrances. Des études d'histologie et d'immunohistochimie sur des coupes coronales pourraient ensuite être effectuées, afin de déterminer la taille et l'hétérogénéité de la tumeur par des méthodes de coloration (hématoxyline et éosine), l'activité proliférative (immunomarquage avec un antigène Ki67) [16], et la présence de cellules apoptotiques (test TUNEL [terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling]) [17].

En conclusion, bien que l'augmentation de la survie des animaux traités avec des nanovecteurs chargés en FcdiOH reste encore modeste, les gains obtenus sont prometteurs à l'égard d'une chimiothérapie du glioblastome qui représente la forme la plus agressive des gliomes. En effet, le traitement de première ligne du glioblastome en clinique, combinaison de la radiothérapie focale à la chimiothérapie avec le Témozolomide, présente également une augmentation de 20% de la médiane de survie des patients par rapport à la radiothérapie seule (passant de 12.1 à 14.6 mois) [18]. Ce travail de thèse a contribué à confirmer l'activité anticancéreuse *in vivo* du FcdiOH, composé organométallique innovant, en mettant en évidence l'influence de la dose et de la voie d'administration sur son efficacité dans un modèle de gliosarcome 9L intracérébral. Par conséquent, il semble capital de poursuivre des études approfondies sur ces nanovecteurs afin d'aboutir à un traitement plus efficace encore permettant d'envisager un passage futur en essai clinique.

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2008-2011 : Doctorat en Pharmacologie expérimentale et clinique
Ecole doctorale de Biologie Santé
Université d'Angers, France

Oct 2010: Formation à l'expérimentation animale Niveau 1,
ONIRIS, Ecole Nationale Vétérinaire, Agroalimentaire et de l'Alimentation
Nantes-Atlantique, France

2004-2005: Master 2 Recherche Mention Science des matériaux et chimie organique
Spécialité chimie organique
UFR Sciences, Université de Caen – Basse Normandie, France.

1999-2004: Diplôme d'état de Pharmacien
Université des Sciences Médicales de Hochiminh ville, Viet Nam

EXPERIENCE PROFESSIONELLE

Sep 2008 à ce jour: thèse de doctorat
Sujet : "*Application des nanocapsules lipidiques chargées en principe actif anticancéreux dans le traitement du gliome*"
INSERM U646, Ingénierie de la vectorisation particulaire
Université d'Angers – France

Depuis Oct 2005: Enseignante/Chercheur
Département de Pharmacologie, Faculté de Pharmacie
Université de Médecine et de Pharmacie de Hochiminh ville – Vietnam

Jan - Juin 2005: Stage pratique de 6 mois du Master 2 Recherche (Pr. Daniel Ladurée)

Sujet : "*Synthèse d'inhibiteurs mixtes dirigés contre la transcriptase inverse et l'intégrase du V.I.H*"

CERMN, centre d'études et de recherches sur le médicament de Normandie

Université de Caen - Basse Normandie – France

Mars-Juillet 2004: Stage pratique validant Diplôme universitaire de Pharmacie

Sujet : "*Étude de l'effet hypolipidémiant de certains produits d'origine naturelle*"

Departement de Pharmacologie, Faculté de Pharmacie

Université des Sciences Medicales de Hochiminh ville – Viet Nam

ACTIVITES SCIENTIFIQUES

Communications orales

Various routes of administration for the treatment of 9L rat gliosarcoma by ferrociphenol-loaded lipid nanocapsules

Huynh N.T., Lemaire L., Vessieres A., Jaouen G., Garcion E., Benoit J.P., Passirani C.

XXVièmes Journées Scientifiques du G.T.R.V., Toulouse, Décembre 2010

Mesenchymal stromal cells as cellular vehicles of nanoparticles: a new therapeutic tool for brain tumors.

Roger M., Clavreul A., Huynh T., Passirani C., Schiller P., Vessières A., Montero-Menei C., Menei P.

XXVièmes Journées Scientifiques du G.T.R.V., Toulouse, Décembre 2010

Communications écrites

Systemic treatment of ferrocenyl-based drug loaded lipid nanocapsules on a subcutaneous 9L gliosarcoma in rats

Huynh N.T., Passirani C., Morille M., Allard E., Vessieres A., Jaouen G., Benoit J.P.

7th world meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology, Malta, Mars 2010

In vivo neuronal toxicity of concentrated lipid nanocapsule suspension administered by convection-enhanced delivery

Huynh N.T., Passirani C., Allard E., Lemaire L., Benoit J.P.

XXIVièmes Journées Scientifiques du G.T.R.V., Paris, Décembre 2009

Development of a long-term hyperlipidemic model induced by tyloxapol in mice
Pham Thi Tuyet Trinh; Huynh Ngoc Trinh, Mai Phuong Mai
The Sixth Indochina Conference on Pharmaceutical Sciences, Hue, Viet Nam, Novembre 2009

Hypolipidemic effect of extracts from *Abelmoschus esculentus* L. – Malvaceae on tyloxapol - induced hyperlipidemia in mice.
Huynh Ngoc Trinh, Nguyen Ngoc Quynh, Tran T Van Anh, Vo Phung Nguyen
The Fifth Indochina Conference on Pharmaceutical Sciences, Bangkok, Thai Lan, Novembre 2007

Acute toxicity and analgesic activity of vietnamese *Lychas mucronatus* scorpion venom in mice.
Nguyen Thi Phuong Khue, Nguyen Nhu Ho, Vo Phung Nguyen, Huynh Ngoc Trinh, Nguyen Hoang Anh
The Fifth Indochina Conference on Pharmaceutical Sciences, Bangkok, Thai Lan, Novembre 2007

Alpha-glucosidase inhibition of charantin from M*omordica charantia* (Cucurbitaceae) in alloxan- induced diabetic mice.
Mai Phuong Mai, Nguyen Thi Mai Hoang, Huynh Ngoc Trinh, Phung Van Trung.
The Fifth Indochina Conference on Pharmaceutical Sciences, Bangkok, Thai Lan, Novembre 2007

Study of antihyperlipidemic activity of Cao Bang bitter tea (*Ilex kaushue s.y.hu* – Aquifoliaceae) leaves extract against tyloxapol induced hyperlipidemia in mice.
Mai Phuong Mai, Huynh Ngoc Trinh
The Fourth Indochina Conference on Pharmaceutical Sciences, Hochiminh ville, Viet Nam, Novembre 2005.

Publications

Fossey C, Huynh NT, Vu AH, Vidu A, Zarafu I, Laduree D et al.: Synthesis and anti-HIV evaluation of hybrid-type prodrugs conjugating HIV integrase inhibitors with d4t by self-cleavable spacers containing an amino acid residue. J Enzyme Inhib Med Chem 22(5), 608-619 (2007)

Huynh NT, Nguyen NQ, Tran TVA, Vo PN. Hypolipidemic effect of extracts from *Abelmoschus esculentus* L. – Malvaceae on tyloxapol - induced hyperlipidemia in mice. Mahidol University Journal of Pharmaceutical Sciences 35(1-4): 42-46 (2008)

<u>En rapport avec ce travail :</u>

Allard E, Huynh NT, Vessieres A, Pigeon P, Jaouen G, Benoit JP et al.: Dose effect activity of ferrocifen-loaded lipid nanocapsules on a 9L-glioma model. Int J Pharm 379(2), 317-323 (2009)

Huynh NT, Passirani C, Saulnier P, Benoit JP: Lipid nanocapsules: a new platform for nanomedicine. Int J Pharm 379(2), 201-209 (2009)

Huynh NT, Roger E, Lautram N, Benoit JP, Passirani C: The rise and rise of stealth nanocarriers for cancer therapy: passive versus active targeting. Nanomedicine (Lond) 5(9), 1415-1433 (2010)

Huynh NT, Passirani C, Allard-Vannier E, Lemaire L, Roux J, Garcion E, Vessieres A, Benoit JP : Administration-dependent efficacy of ferrociphenol lipid nanocapsules for the treatment of intracranial 9L gliosarcoma in rats. *Accepted in International Journal of Pharmaceutics (2011)*

Huynh NT, Morille M, Bejaud J, Legras P, Vessieres A, Jaouen G, Benoit JP, Passirani C : Treatment of 9L gliosarcoma in rats by ferrociphenol loaded lipid nanocapsules based on passive targeting strategy through EPR effect. *Submitted for publication in Pharmaceutical Research*

Huynh NT, Laine AL, Claveul A, Bejaud J, Legras P, Vessieres A, Eyer J, Benoit JP, Passirani C : An active targeting strategy through ferrociphenol-loaded lipid nanocapsules for the treatment of 9L rat gliosarcoma. *Submitted for rapid communication in Eur J Pharm Biopharm*

<u>En parallèle à ce travail :</u>

Roger M, Clavreul A, Huynh NT, Passirani C, Schiller PC, Vessières A, Montero-Menei CN, Menei P: Ferrociphenol loaded lipid nanocapsules deliver by mesenchymal stem cells in brain tumor therapy. *Submitted in International Journal of Pharmaceutics (minor corrections)*

Résumé

Ce travail a pour but d'optimiser la chimiothérapie du glioblastome à l'aide de nanocapsules lipidiques (LNC) chargées en ferrociphénol (FcdiOH), un composé organo-métallique anticancéreux innovant. Différentes voies d'administration ont été envisagées : la voie locale par stéréotaxie (convection-enhanced delivery ou CED) (60 µL de LNC, 0.36 mg de FcdiOH/rat), l'injection intra-carotidienne et l'injection intraveineuse (400 µL de LNC, 2.4 mg de FcdiOH/rat). Sur le modèle 9L orthotopique, l'efficacité antitumorale du principe actif s'est avérée être proportionnelle à la dose injectée. Le traitement local par CED d'une suspension iso-osmolaire de LNC-FcdiOH a augmenté significativement la survie des rats traités par rapport à celle du groupe témoin (médiane de 28.5 jours au lieu de 25 jours). Le recouvrement par de longues chaines de poly(éthylène glycol) (DSPE-mPEG2000) a permis aux LNC ainsi pégylées d'améliorer leur temps de circulation sanguine avec l'obtention d'une demi-vie 4 fois plus longue et d'une aire sous la courbe 1.65 fois plus étendue que celles des LNC classiques. Cela a entraîné l'éradication de la tumeur 9L sous-cutanée après une seule injection intraveineuse de DSPE-mPEG2000-LNC-FcdiOH, montrant l'efficacité du ciblage passif obtenu avec ces nanovecteurs. En parallèle, l'injection intra-carotidienne représente une voie d'administration prometteuse pour la délivrance de principes actifs dans le cerveau. En effet, le traitement intra-carotidien à l'aide des LNC pégylées a permis d'augmenter de 20% la survie des rats porteurs d'un gliosarcome 9L intracérébral (médiane de 30 jours). Enfin, l'incorporation de peptides NFL-TBS à la surface des LNC semble être une approche intéressante dans le cadre d'un ciblage actif, des études préliminaires ayant mis en évidence un rat survivant jusqu'à 44 jours.

Mots clés : gliosarcome, convection-enhanced delivery (CED), injection intra-carotidienne, poly(éthylène glycol), peptide NFL-TBS, ciblage passif, ciblage actif.

Abstract

This thesis work aims at optimizing the chemotherapy of glioblastoma by lipid nanocapsules (LNC) loaded with an innovant organometallic anticancer drug, the ferrociphenol (FcdiOH). To this end, various routes of administration for drug delivery were investigated: convection-enhanced delivery (CED) (60µL LNC, 0.36mg of FcdiOH/rat), intra-carotid injection and intravenous injection (400µL LNC, 2.4mg of FcdiOH/rat). On 9L orthotopic model, antitumoural effect of FcdiOH exhibited a dose dependant effect. The local administration by CED of an iso-osmolar suspension of FcdiOH-LNC significantly increased the median survival time of treated rats as compared to untreated controls (28.5 days *versus* 25 days). The coating with longer chains of poly(ethylene glycol) (DSPE-mPEG2000) allowed improving the blood circulation time of such pegylated LNC with 4-fold longer half-life and 1.65-fold larger area under the curve than these of classic ones. This leaded to the eradication of subcutaneous tumour after a single intravenous injection of DSPE-mPEG2000-FcdiOH-LNC, showing the efficacy of passive targeting with these nanovectors. In addition, the intracarotid injection represents a promising route of administration for drug delivery to the brain. Interestingly, the intracarotid treatment with DSPE-mPEG2000-FcdiOH-LNC dramatically prolonged the survival time of intracranial 9L-tumour-bearing rats (median of 30 days). Finally, the incorporation of NFL-TBS peptide at the LNC surface seems to be an interesting approach through the active targeting, as preliminary studies evidenced one rat surviving until day 44.

Keyword: gliosarcoma, convection-enhanced delivery (CED), intra-carotid injection, poly(ethylene glycol), NFL-TBS peptide, passive targeting, active targeting.