Etudes précliniques sur la radiosensibilisation des tumeurs gliales de haut grade par chimiothérapie locale encapsulée.

Sandrine Vinchon-Petit

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par : Sandrine VINCHON-PETIT

Devant le jury ci-dessous :

François-Régis BATAILLE (Président), Professeur, Université d'Angers.

Jean-Jacques MAZERON (Rapporteur), Professeur, Université de Paris VI.

Benoît BATAILLE (Rapporteur), Professeur, Université de Poitiers.

Emmanuel GARCION (Invité), INSERM U646, Angers.

Directeur de Thèse:

Philippe MENEI, Professeur, Université d'Angers

Nom et Coordonnées du Laboratoire :

Laboratoire INSERM U646,

10, rue André Boquel 49933 ANGERS Cedex 9. Tél : 02 41 73 58 55

COMPOSITION DU JURY:

PRESIDENT

Monsieur le Professeur François-Régis BATAILLE

DIRECTEUR DE THESE:

Monsieur le Professeur Philippe MENEI

RAPPORTEURS :

Monsieur le Professeur Jean-Jacques MAZERON

Monsieur le Professeur Benoît BATAILLE

EXAMINATEURS :

Monsieur le Professeur François-Régis BATAILLE

Monsieur Emmanuel GARCION (Invité)

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Professeur de Neurochirurgie à la Faculté de Médecine d'Angers

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Directeur du Centre Régional de Lutte Contre le Cancer Paul Papin

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ABREVIATIONS

ADN	Acide DésoxyriboNucléique
AMM	Autorisation de Mise sur le Marché
ARN	Acide RiboNucléique
BHE	Barrière Hémato-Encéphalique
CED	Convection-Enhanced Delivery
Cm	centimètre
EORTC	European Organisation for Research and Treatment of Cancer
Gy	Gray
IRM	Imagerie par Résonnance Magnétique
IV	Intra-veineuse
LNC	Nanocapsules lipidiques
MDR	Multidrug Resistance
ml	millilitre
mm	millimètre
m/m	masse pour masse
NCI	National Cancer Institute
P-gp	Glycoprotéine P
PVA	PolyVinyle Acid
RTE	Radiothérapie externe
RTOG	Radiation Therapy Oncology Group
SNC	Système Nerveux Central
TMZ	Témozolomide
µg	microgramme
µl	microlitre

1. INTRODUCTION

1.1. Les tumeurs gliales malignes: épidémiologie, pronostic et évolution.

Avec 3000 nouveaux cas par an en France, le glioblastome représente la tumeur cérébrale primitive la plus fréquente et son incidence ne cesse d'augmenter. Son pronostic reste effroyable avec une médiane de survie comprise entre 10 et 15 mois malgré un traitement complet associant une chirurgie, dont la qualité est un facteur de survie important, la radiothérapie et la chimiothérapie (1, 2).

La prise en charge des glioblastomes représente un challenge pour les neuro-oncologues. Le glioblastome possède des propriétés infiltrantes et présente une nature hautement invasive (Figure 1). La prise de contraste bordant la lésion tumorale à l'IRM ne constitue pas la limite de cette tumeur. Les biopsies pratiquées plusieurs centimètres au-delà des marges délimitées par l'IRM, dans les zones macroscopiquement saines, retrouvent des cellules tumorales (3).

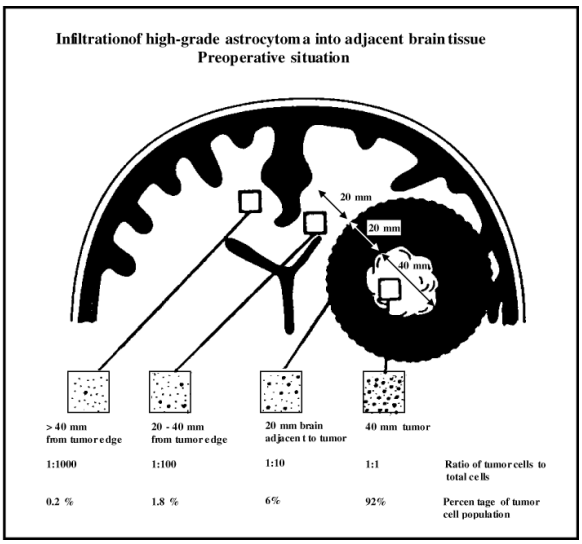


Figure 1: Représentation des propriétés infiltrantes des tumeurs gliales de haut grade (en situation pré-opératoire).

Cependant, l'expérience clinique va à l'encontre de ces données. Malgré une résection macroscopique la plus complète possible, 90% des récidives surviennent dans un rayon de 2 cm autour du site tumoral initial (4-7). Ainsi, cibler les cellules tumorales résiduelles considérées comme les plus agressives, tout en protégeant le tissu cérébral environnant constitue un objectif

majeur. Toutefois, la fragilité du tissu cérébral sain adjacent et la présence de la barrière hémato-encéphalique (BHE) limitent les possibilités thérapeutiques classiques qu'elles soient chirurgicales, radiothérapiques ou chimiques.

Parallèlement, l'escalade de doses requise par l'utilisation de la voie veineuse est rapidement limitée par la toxicité systémique qu'elle entraîne (ex : thrombopénie et nitrosurées).

Ainsi, les stratégies d'utilisation locale d'agents cytotoxiques au sein du SNC permettent de s'affranchir de ces inconvénients.

1.2. Les thérapies locales.

L’histoire des thérapies locales a commencé par l’adaptation du vieux concept de la brachythérapie dans la prise en charge des tumeurs cérébrales. Initialement, des implants d’iode radioactif pouvaient être déposés au sein du parenchyme cérébral. Cependant, aucune étude de phase II n'a vu le jour. Puis, des techniques plus sophistiquées ont vu le jour tel que le Gliasite mais n'ont pas été développées par la suite (Figure 2).

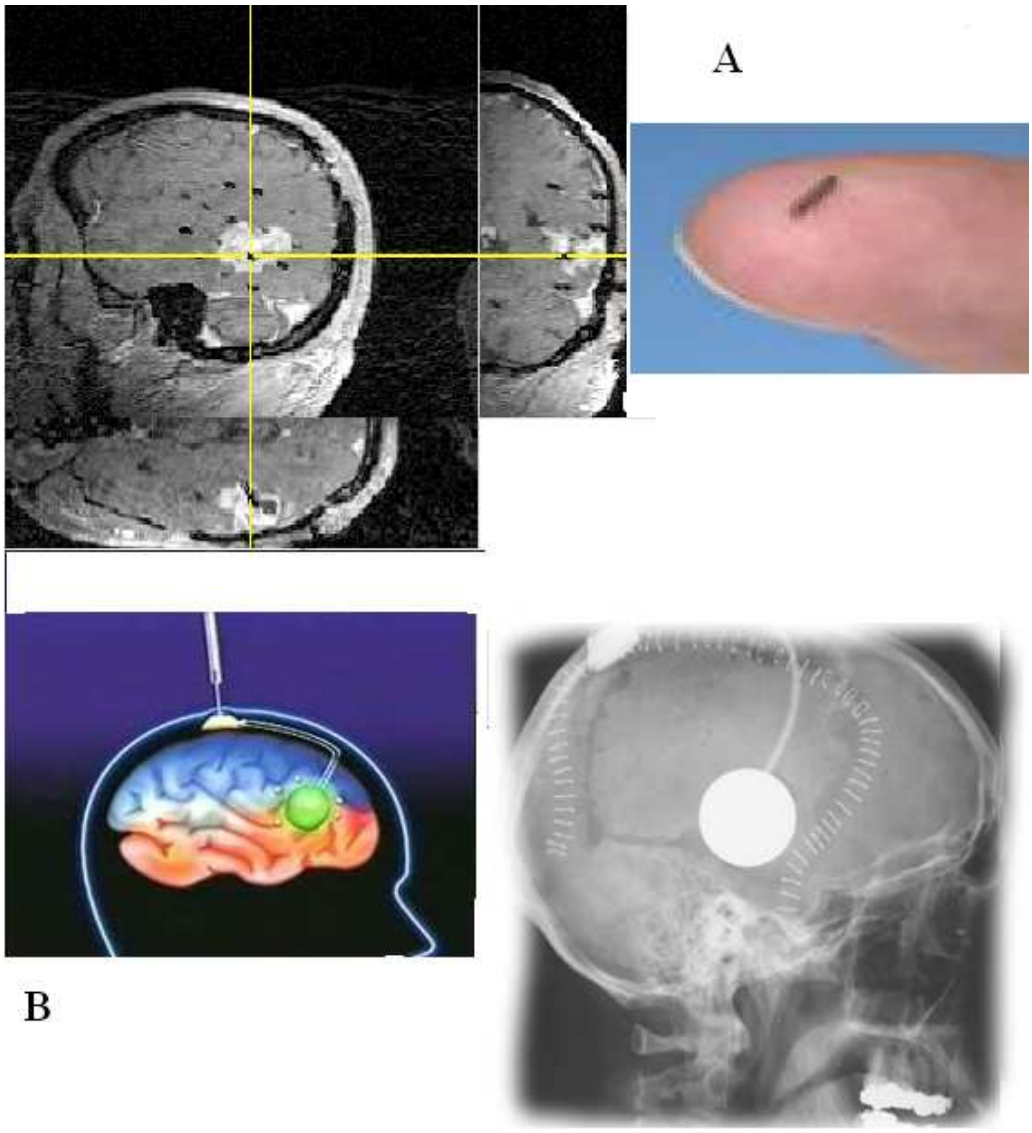


Figure 2: Brachythérapie adaptée aux tumeurs cérébrales sous forme d’implants d’iode radioactif (A) ou de cathéter intracrânien délivrant de l’iode radioactive sous forme liquide (Gliasite®) (B).

D’autres voies ont été explorées pour l’administration locale d’agents anticancéreux:

✓ **L’injection directe intraparenchymateuse** générant un gradient de pression (Photo 1). Ce type d’injection entraîne très souvent la survenue d’un reflux du produit injecté vers la surface. La concentration du principe actif finalement obtenue dans la cible est donc limitée. Ce procédé n’est donc pas la technique de choix pour l’injection locale de solution.

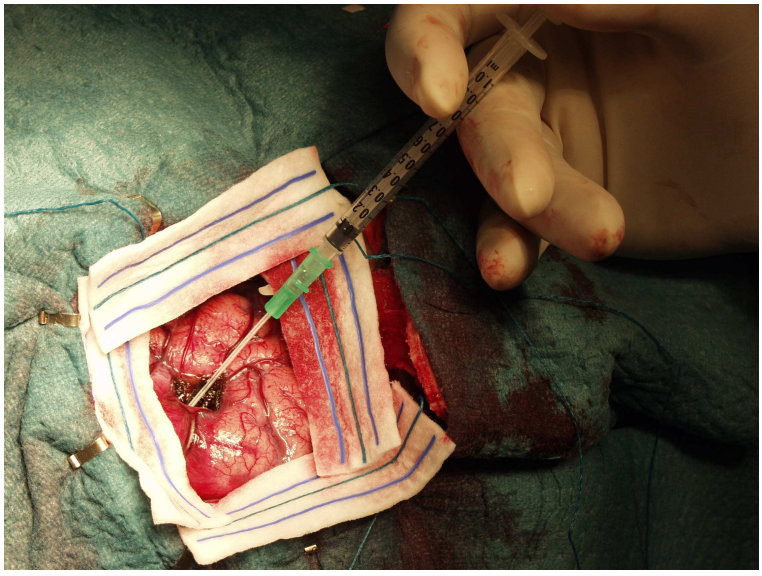
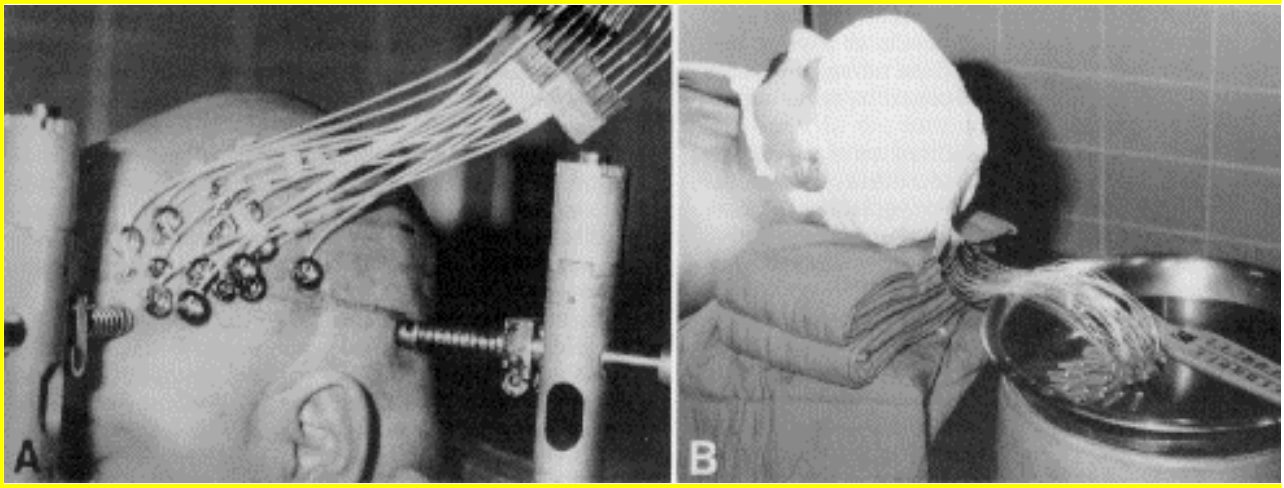


Photo 1: Injection locale directe intra-parenchymateuse cérébrale à la seringue d’un soluté en per opératoire.

✓ **La Convection-Enhanced Delivery (CED)** permet de délivrer l’agent thérapeutique pendant qu’est maintenu un gradient de pression continu et déterminé au préalable. Ce gradient est imposé par une pompe osmotique. Il permet de maintenir une pression interstitielle entraînant une propagation («bulk flow») du produit injecté vers les espaces périvasculaires, au travers du compartiment extracellulaire (8) (Photos 2A et B), (Figure 3).



Photos 2A et B: Illustration d'un montage de Convection-Enhanced Delivery en pratique clinique. A: Implantation au sein du parenchyme cérébral de cathéters. B: Montage reliant les cathéters au système de pompe à l'aide de tubulures étanches.

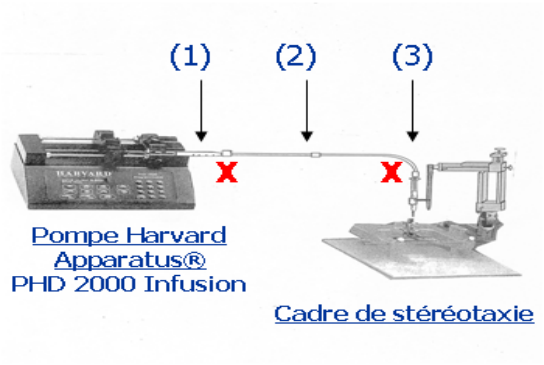


Figure 3: Représentation d'un montage de Convection-Enhanced Delivery en pratique préclinique. Adaptation sur le cadre de stéréotaxie du matériel d'injection relié à la pompe.

✓ **<u>La thérapie cellulaire</u>**, encore appelée biothérapie, implique des cellules génétiquement modifiées. Ces cellules ont acquis la propriété de synthétiser des protéines anti-tumorales ou de libérer des particules virales possédant un effet thérapeutique (9). Elles peuvent être inoculées au sein du parenchyme cérébral. Elle constitue une approche prometteuse mais qui est encore en développement. Bien qu'elle représente une modalité de thérapie locale, elle ne permet pas, à

proprement parler, l'administration d'agent chimique thérapeutique. Ainsi, même s'il était important de la mentionner, cette voie ne sera pas abordée dans ce travail.

✓ **Les implants polymériques** permettent une diffusion lente et contrôlée du principe actif au sein du tissu cérébral.

a. Les implants polymériques

Les premiers implants utilisés en pratique clinique datent des années 60. Ils étaient constitués d'une simple éponge de gélatine imbibée d'endoxan ou de méthotrexate. Ils étaient posés simplement dans la cavité opératoire (Photo 3). Des neurochirurgiens japonais ont été les premiers à utiliser de vrais implants polymériques pour traiter des tumeurs cérébrales. Dans plusieurs études, ils ont travaillés avec des polymères non biodégradables délivrant des nitrosourées (ACNU), du 5-FU ou de l'adriamycine (10-12). Malheureusement, ces études étaient juste des études pilotes et les auteurs n'ont pas pu conclure à leur efficacité.

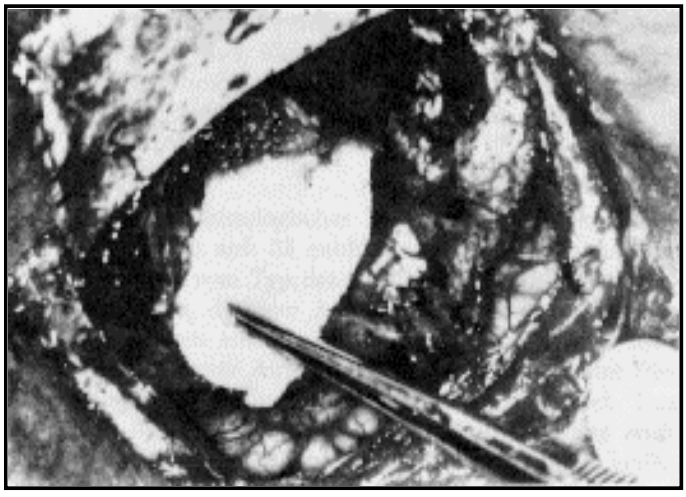


Photo 3: Première application clinique des éponges de gélatine imbibées d'agents cytotoxiques: implantation intra-cavitaire d'une éponge de gélatine en per-opératoire.

Récemment, des micro-technologies plus sophistiquées ont été mises au point. Maintenant, les implants utilisés sont la plupart biodégradables. Ils protègent l'agent

thérapeutique jusqu'à sa cible et permettent ensuite sa libération lente et contrôlée. Le seul implant polymérique qui a démontré son efficacité contre les tumeurs gliales de haut grade dans des essais de phase III est le Gliadel®. Il est composé d'un copolymère de la famille des polyanhydrides contenant des molécules de p-cpp et des molécules d'acide sébacique, liées par une liaison covalente qui peut être cassée par hydrolyse (Figure 4). Ainsi, l'eau constitue le seul élément nécessaire à la dégradation de l'implant et donc à la libération de la chimiothérapie. A l'origine, ce copolymère était destiné à l'industrie du textile afin de remplacer le polyester. Il a été rapidement abandonné du fait de cette «instabilité» chimique le rendant facilement dégradable. C'est aujourd'hui son point fort pour son application en médecine. Il représente à ce jour la seule formulation ayant obtenue une autorisation de mise sur le marché pour apposition (8 implants maximum) dans la cavité de résection après exérèse chirurgicale d'un gliome malin, qu'il soit nouvellement diagnostiqué ou pour les patients en récidive lorsqu'une intervention chirurgicale est possible (13-16) (Photo 4).

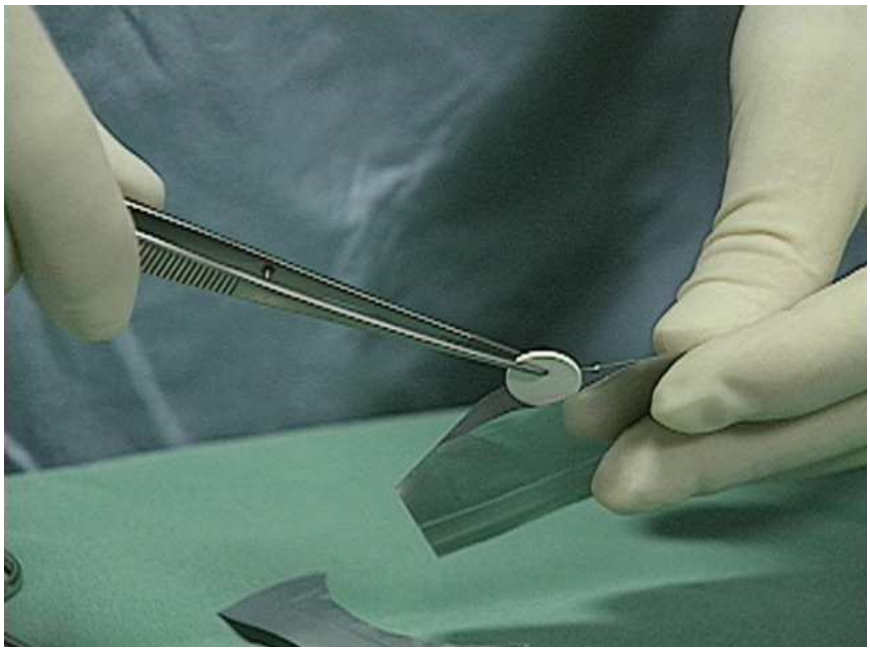


Photo 4: Implant de Gliadel®**.**

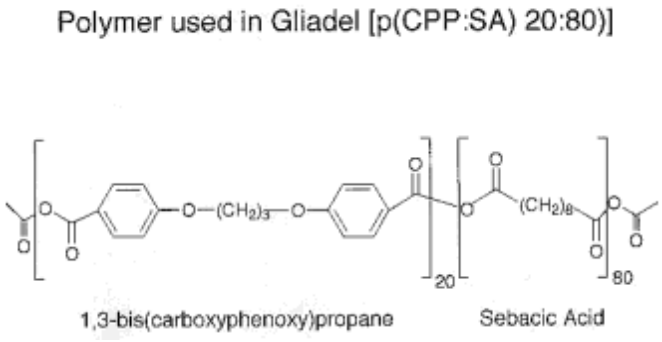


Figure 4: Formule chimique de l'acide poly (1,3-bis (carboxyphénoxy) propane-co-sabacique (Polifeprosan®), polymère utilisé dans la formulation du Gliadel®.

b. Microsphères

Bien que de nombreux systèmes de libération aient été développés et évalués sur des modèles animaux, très peu ont amené la mise en place d'essais cliniques de phase I (17). Parallèlement à ces implants monolithiques, l'Inserm U646 a mis au point des microsphères de copolymère d'acide lactique-co-acide glycolique libérant un antimétabolite radiosensibilisant, le 5-Fu (5-fluorouracile) sur plus d'un mois (18-26) (Schéma 1). La taille de ces microparticules (1 à 1000 µm) permet de les injecter de façon précise, même dans les zones cérébrales fonctionnelles, sous la forme d'une suspension, à main levée ou par stéréotaxie sans léser le tissu cérébral (18) (Photo 5). La faisabilité de l'implantation au niveau de la cavité de résection chirurgicale a été démontrée par des études de phase I/II pour les glioblastomes opérables (27) mais également au sein de la tumeur pour les tumeurs cérébrales profondes non opérables (28). Une étude de phase IIb (microsphères de 5-Fu associées à la radiothérapie versus radiothérapie seule) a démontré un bénéfice non statistiquement significatif en termes de survie (27-28).

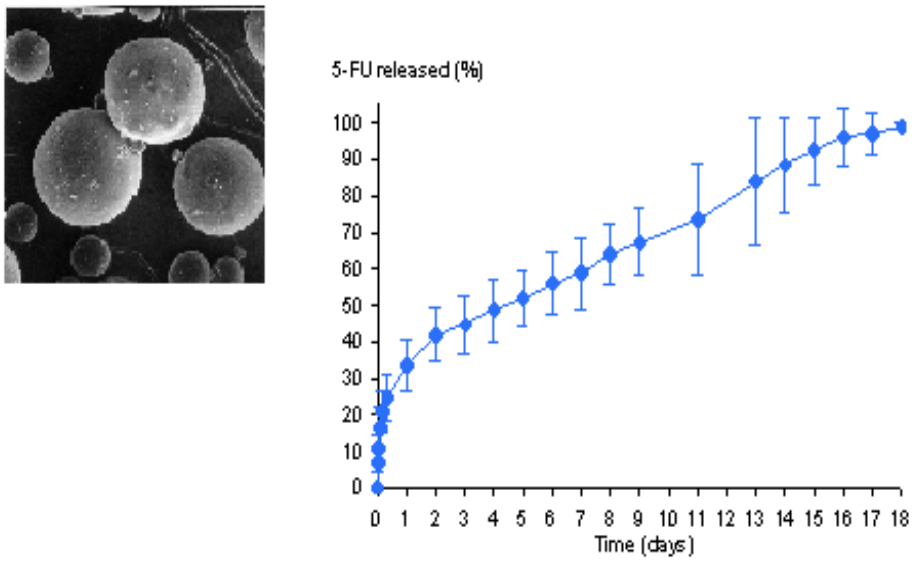


Schéma 1: Cinétique de libération du 5-FU par les microsphères.

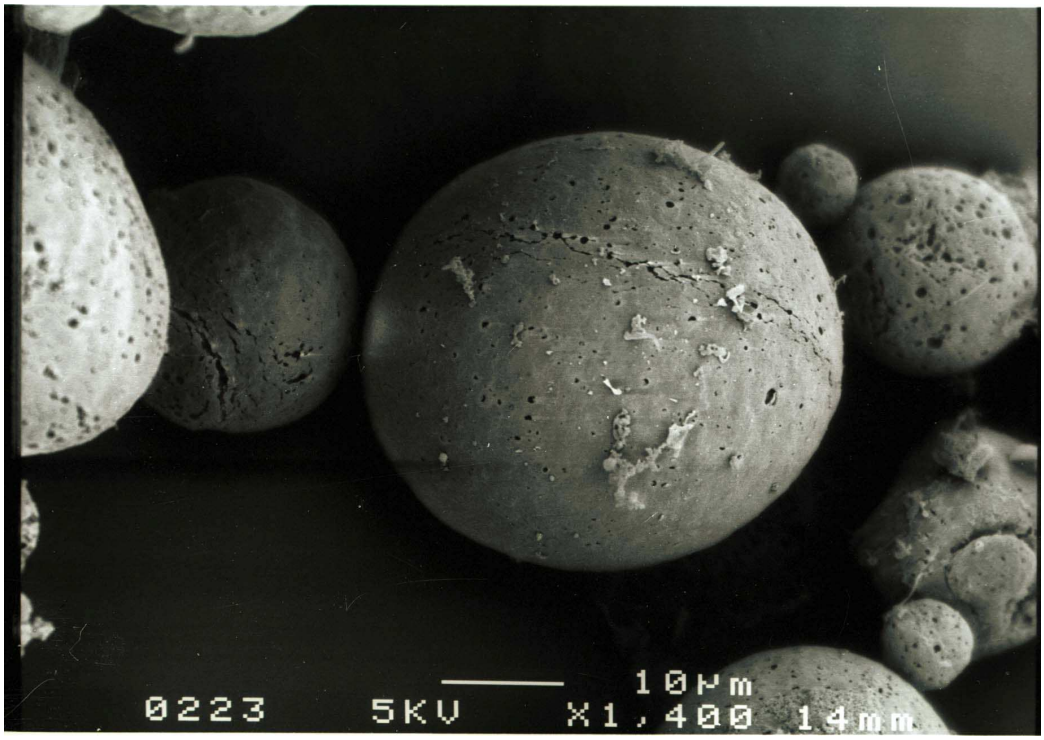


Photo 5: Microsphères de 5-FU en suspension.

Pour ces systèmes polymères, l´efficacité de la chimiothérapie libérée est réduite par une diffusion limitée à partir des implants. Ils favorisent l'obtention d'un index thérapeutique élevé mais dans un volume tissulaire tumoral qui reste limité (29).

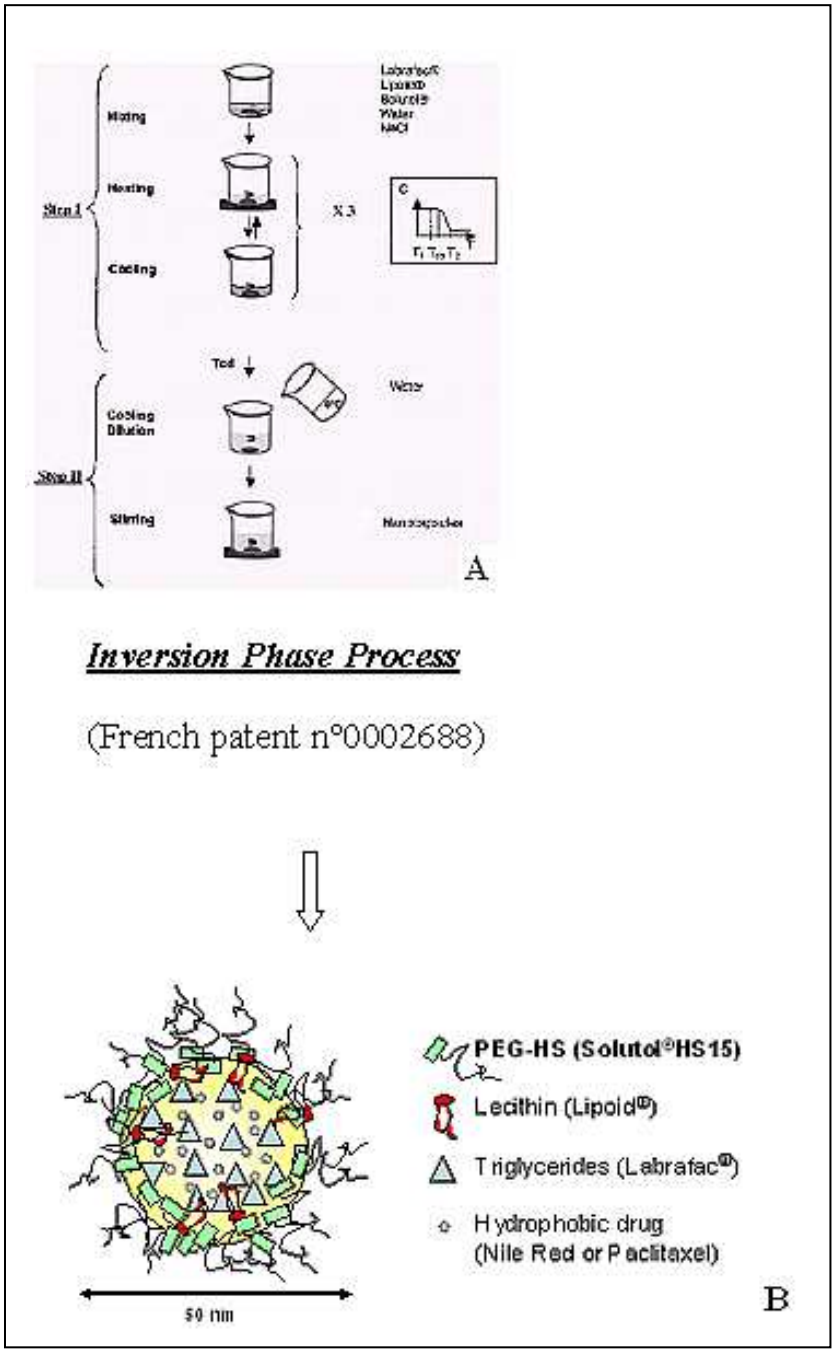
Aussi, les outils thérapeutiques envisagés se heurtent encore largement au caractère échappatoire des gliomes, qui peut être associé à l'expression élevée de protéines de survie telles que la protéine Bcl-2, à l'expression de transporteurs d'efflux participant au phénomène de MDR (résistance "multidrug"), ou à un coefficient de prolifération abaissé pouvant notamment entraîner une insensibilité à des doses d'irradiation inférieures à 60 grays (30).

La récente expansion des nanotechnologies - qu'il s'agisse de liposomes, de nanosphères polymériques, de nanocapsules polymériques ou nanoparticules lipidiques- associée aux progrès des méthodes stéréotaxiques d'administration intracérébrale de médicaments offre l'opportunité d'améliorer l'index thérapeutique des principes actifs libérés. Le devenir du principe actif n'est alors plus dépendant de ses propriétés intrinsèques mais de celles de son vecteur. Parallèlement, il est protégé du reste de l'organisme jusqu'à sa libération, si possible à proximité de sa cible, pour plus d'efficacité, de spécificité et de sécurité biologique

c. Nanoparticules

Par une méthode d'émulsion par inversion de phase sans utilisation de solvant organique, l'Inserm U646 a récemment développé et breveté des nanocapsules lipidiques (LNC) (31) (Figures 5A et B). Ces LNC, dont la taille peut être ajustée de 20 à 100 nm avec une distribution monodisperse, sont capables de cibler le compartiment intracellulaire des cellules cancéreuses de gliomes et de servir de réservoir à partir duquel peut être libéré un agent anticancéreux tel que le paclitaxel, tout en le rapprochant de sa cible intracellulaire, la ß-tubuline. De plus, une interaction entre les LNC et des pompes d'efflux a été mise en évidence aboutissant à une inhibition de la MDR dans les cellules de gliome en culture et implantées chez l'animal. Il a été établi que les LNC chargées en paclitaxel sont plus efficaces que le paclitaxel sous une de ses

formulations disponibles en clinique, le Taxol®, en augmentant la mort des cellules cancéreuses *in vitro* et en réduisant l'expansion tumorale des gliomes implantées en sous-cutané *in vivo* (32).



Figures 5A et B: Procédé de fabrication des nanocapsules lipidiques (LNC).

Ces différents vecteurs et ces différentes techniques d'implantation, résumés dans la figure 6, font l'objet de nombreuses recherches précliniques en vue de démontrer leur efficacité mais également dans le but d'adapter leur utilisation à la pratique clinique.

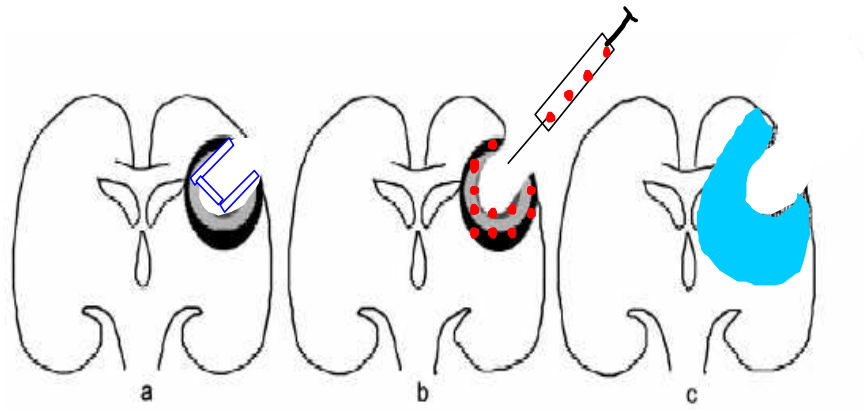


Figure 6: Représentation schématique des différents systèmes de thérapie locale et leur diffusion. a: Implants monolithiques de Gliadel ®; b: Injection de microsphères de 5-Fu; c: Injection de substances en solution par CED.

✓ **Article n°1**

- Chimiothérapie locale dans les gliomes malins: de l'injection à la seringue aux nanotechnologies.
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Chimiothérapie locale dans les gliomes malins : de l'injection à la seringue aux nanotechnologies

Local chemotherapy of malignant glioma: From syringe injections to nanotechnology

S. Petit [a,b], E. Garcion [a], J.-P. Benoit [a], P. Menei [a,c,*]

[a] Inserm U646, Angers, France
[b] Centre Paul-Papin, Angers, France
[c] Département de neurochirurgie, CHU d'Angers, 4, rue Larrey, 49933 Angers cedex 9, France

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RÉSUMÉ

De nombreux arguments sont en faveur du développement de thérapies locales dans les gliomes malins. Les simples injections d'antimitotiques dans la cavité opératoire ont laissé place à des systèmes sophistiqués de perfusion tissulaire, à des systèmes polymères ou lipidiques à libération prolongée, macro-, puis microscopiques et maintenant nanométriques. Mais comme pour tout médicament, les développements sont longs et peu ont atteint le stade de la clinique.

ABSTRACT

Many arguments support the development of local therapies for malignant gliomas. Simple injections of antimitotic agents into the surgical cavity has been replaced by more sophisticated systems. Tissues can be infused with complex prolonged-release polymeric or lipidic systems with macroscopic, microscopic and now even nanometric particles. But, as for any drug, the developments of these new agents has been long and only very few reach the stage of the clinic trials.

1. Introduction

Les gliomes malins, regroupant des entités aussi différentes que le glioblastome, l'oligodendrogliome anaplasique et les oligoastrocytomes anaplasiques, sont des tumeurs au comportement assez déroutant. Il est bien démontré que la prise de contraste sur l'IRM est loin de représenter la vraie limite de ces tumeurs hautement infiltrantes. Des biopsies réalisées en zones macroscopiquement saines, plusieurs centimètres en dehors des limites IRM de la tumeur,

* Auteur correspondant.
Adresse e-mail : PhMenei@chu-angers.fr (P. Menei).
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permettent d'isoler des cellules tumorales (Sibergeld et Chicoine, 1997). Il est donc permis de se demander si le gliome malin n'est pas une maladie globale du cerveau... Paradoxalement, la simple observation clinique va à l'encontre de ce caractère global de la maladie. En effet, le mode de présentation des gliomes malins est généralement unifocal, les dissémination sous-arachnoïdiennes, même après une évolution prolongée, sont rares. De même, les métastases extraneurales sont exceptionnelles. Enfin, et c'est l'élément d'observation le plus frappant, 90 % de ces tumeurs récidivent dans les 2 cm du site de résection chirurgicale (Liang et al., 1991 ; Wallner et al., 1989). On peut donc conclure que si le gliome malin n'est pas une maladie locale, il se comporte comme tel ou au moins comme une maladie locorégionale. Un traitement local n'est donc pas illogique.

Plusieurs arguments peuvent être avancés en faveur d'un traitement in situ des gliomes malins. Le premier est carcinologique : la limitation de dose imposée en radiothérapie ne permet pas l'obtention d'un contrôle local. Une administration in situ d'anticancéreux pourrait favoriser ce contrôle local et donc augmenter le délai sans récidive et peut-être la survie globale. Les autres arguments sont d'ordre pharmacologique. Une administration locale permet d'améliorer l'index thérapeutique in situ, sans augmentation des taux circulants et donc sans majoration de la toxicité systémique. Ce point est particulièrement intéressant pour les antimitotiques conventionnels dont la toxicité est essentiellement hématologique. Cette absence de toxicité générale autorise une administration longue et continue. Ce maintien prolongé d'une concentration efficace est important, car un grand nombre de substances exerce leur action à une phase précise du cycle cellulaire. Aussi, l'absence de toxicité systémique, qui, il faut l'avouer, se fait souvent au dépend d'une toxicité nerveuse locale, permet d'associer ces thérapeutiques locales aux thérapies systémiques.

Le dernier argument enfin, est représenté par la barrière hématoencéphalique. Si cette barrière est généralement altérée au niveau du centre de la tumeur, elle est intacte à sa périphérie, là où des cellules malignes ont migré dans le tissu sain (Donelli et al., 1992). L'administration locale est la seule possible pour les grosses molécules (anticorps, protéines, complexes oligonucléotidique), qui ne passent pas cette barrière et parmi lesquelles se trouvent les derniers médicaments développés contre les gliomes.

Cette revue se limitera à l'administration d'antimitotiques conventionnels. Toutefois, il faut savoir qu'actuellement, de nombreux produits issus des biothérapies, également administrés localement, sont en essai de phase III, comme par exemple molécules chimères associant un ligand à une toxine bactérienne ou adénovirus porteurs de gène tueur. Il est d'ailleurs étonnant de constater que les modes d'administration ont peut évolué par rapport à la complexité des produits administrés, et que les produits de thérapie génique les plus sophistiqués sont encore administrés par une injection à la seringue de 1 ml dans les parois de la cavité de résection.

Des méthodes plus sophistiquées d'administration locale ont récemment vu le jour, comme la perfusion cérébrale par *convection-enhanced delivery* (CED) et les systèmes polymères à libération prolongée. Si de nombreuses expérimentations animales et autres essais cliniques ont été déjà publiés, l'administration locale intracérébrale en est encore à ses débuts. En effet, les laboratoires développant cette approche restent confrontés à des questions d'ordre pharmacologique non complètement résolues. Parmi elles, on peut citer la difficulté à appréhender la diffusion tissulaire in vivo chez l'homme, ainsi que les questions récurrentes à propos des doses à injecter (très différentes des doses administrées par voies parentérale et dont on ne sait pas vraiment encore s'il faut les adapter au patient). L'évaluation clinique pose, elle aussi, des questions méthodologiques nouvelles : comment baser l'augmentation de dose des phases II ? Le groupe de référence doit-il comporter un implant placebo ? Cela est-il éthiquement acceptable ? Peut-on continuer à utiliser « l'intervalle libre sans progression » comme critère principal alors que les thérapies locales modifient l'imagerie ? Bien d'autres questions existent, aboutissant, dans les essais cliniques publiés, à des imperfections méthodologiques liées à la nouveauté et à la difficulté du thème abordé.

2. Administration par *convection-enhanced delivery*

Les premières tentatives d'administration d'antimitotiques, soit dans la tumeur, soit dans la cavité d'exérèse, utilisaient un cathéter dont l'extrémité était extériorisée. Des injections répétées d'ACNU, de BCNU, de bléomycine, de methotrexate ou de dérivés du platine ont été, ainsi, réalisées (Olivi et al., 1993 ; Walter et al., 1995).

La mise en place d'un réservoir sous-cutané (réservoir d'Ommaya) permettant des injections répétées tout en diminuant les risques infectieux a été le premier progrès apporté. Des essais ont été, ainsi, réalisés avec de l'ACNU, de l'adriamycine, de la bléomycine ou du methotrexate (MTX) (Walter et al., 1995 ; Yamashima et al., 1990). Des pompes, elles aussi implantées en sous-cutanée, ont parfois été utilisées comme suit : pompes à débit continu, fonctionnant avec du gaz sous pression ou pompes programmables de type péristaltique. Elles ont permis d'apporter du cisplatine, du 5-FU ou du methotrexate dans les cavités opératoires (Damascelli et al., 1991 ; Nierenberg et al., 1991 ; Walter et al., 1995).

Toutes les études citées ci-dessus sont des rapports préliminaires ou des études pilotes, réalisés sur un petit nombre de patients, quelques fois un seul, et aucune conclusion ne peut malheureusement en être tirée. De plus, la pharmacocinétique de ces types d'administration a été peu étudiée.

Par rapport à ces injections, la *convection-enhanced delivery*, développée en 1994 par Oldfield et al. (Bobo et al., 1994), est un progrès réel pour l'administration dans le cerveau.

La méthode implique le positionnement d'un cathéter au sein du tissu à perfuser. La molécule, diluée dans un fluide, est délivrée grâce à un gradient de pression. Ce gradient est imposé par une pompe osmotique et permet de maintenir une pression interstitielle entraînant une propagation (*bulk flow*) du produit injecté vers les espaces périvasculaires, au travers du compartiment extracellulaire (Bankiewicz et al., 2000). Le volume de distribution peut s'étendre sur plusieurs centimètres, de façon homogène, au-delà de la source, par opposition aux injections intracérébrales simples pour lesquelles le produit reste au mieux localisé au site d'injection ou au pire

reflue (Bankiewicz et al., 2000 ; Krauze et al., 2005 ; Lieberman et al., 1995). Le débit de perfusion ainsi que le volume de solution à injecter peuvent être régulés contrairement aux injections intracérébrales simples. Dans ce dernier cas, le débit trop élevé est responsable de lésions du parenchyme en regard du site d'injection et le volume à injecter doit être limité pour des raisons de tolérance clinique. Lors de la CED, la molécule peut être délivrée à des débits variant de 0,1 à 1 μl/min sans risque de reflux et avec une tolérance satisfaisante (Chen et al., 1999). Le volume infusé peut être adapté comme décrit dans l'étude de Lonser dans laquelle les primates pouvaient recevoir, sous contrôle IRM, des perfusions intra-striatales allant de 2880 à 5760 μl (Lonser et al., 2005).

De nombreux essais précliniques et cliniques de phases I–II confirment la faisabilité et l'innocuité de la CED. Divers agents ont pu être, ainsi, introduits dans le parenchyme cérébral, et ce, dans de multiples pathologies : l'aracytine C dans la leucoencéphalopathie du sujet VIH positif (Levy et al., 2001) ; le vecteur viral AAV2 dans la maladie de Parkinson dans le cadre d'essais sur la thérapie génique (Bankiewicz et al., 2000), de petites molécules comme le topotécan (Kaiser et al., 2000) ou le paclitaxel (Lidar et al., 2004), des nanoparticules magnétiques, des liposomes (Krauze et al., 2005) ou des nanocapsules lipidiques.

3. Systèmes polymères à libération prolongée

Prélude au développement des systèmes polymères, des éponges de matériel hémostatique résorbables, imbibées d'agent cytotoxique comme le méthotrexate, la bléomycine, ou le 5-FU ont été laissées en place dans la cavité opératoire (Walter et al., 1995). L'absence de contrôle de la stabilité de la molécule et de sa cinétique de libération ont logiquement fait abandonner cette approche.

Ce sont les systèmes polymères à libération prolongée, issus de la recherche en ingénierie pharmaceutique, qui ont véritablement ouvert la voie aux thérapies locales. Ces systèmes sont basés sur l'emprisonnement de molécules actives au sein d'un réseau polymérique, qui va les libérer de façon contrôlée et prolongée par des phénomènes de diffusion et/ou percolation. Cette stratégie a été décrite la première fois en 1960 par Folkman et Long qui ont démontré, chez le chien, qu'une pièce de silicone implantée dans le myocarde, pouvait libérer de la digoxine (Folkman et Long, 1964). La libération peut être observée sur une période définie allant de quelques jours à plusieurs mois. Le profil de cette libération peut aussi être programmé, et adapté à la situation. En théorie, toutes les cinétiques peuvent être obtenues, comme par exemple une cinétique de libération d'ordre zéro, en plateau ou biphasique. Les derniers systèmes permettent même de libérer une molécule, puis une autres après un délai programmé. De plus, l'incorporation de la molécule dans un polymère permet de la protéger, apport important pour les molécules instables.

On distingue des systèmes monolithiques allant de quelques millimètres à quelques centimètres et de forme diverse (disque, tube, aiguille...), qui sont mis en place dans la cavité opératoire lors de la craniotomie. Les systèmes polymères particulaires ont une taille comprise entre 1 et 1000 μm. Cette petite taille autorise l'implantation dans le tissu cérébral, en particulier par stéréotaxie (Menei et al., 1994b). Bien qu'un grand nombre de polymères naturels ou synthétiques soient disponibles pour la formulation de ces systèmes, peu sont biocompatibles et souhaitables pour une utilisation clinique.

La première génération de ces systèmes était monolithique et composée de polymères non biodégradables comme l'EVAc ou le silicone, ce qui a, bien sûr, considérablement limité leur utilisation (Langer, 1991 ; Tamargo et al., 1993). Actuellement, les polymères biodégradables sont les plus développés en médecine pour des raisons évidentes. Parmi eux, l'albumine humaine ou bovine, la gélatine, le collagène, l'alginate ou les chitosans, dérivés de carapace de crustacés, ont été utilisés. Mais les problèmes actuels de prions et de sécurité sanitaire ont stoppé leur utilisation. Les polymères synthétiques comme les polyesters aliphatiques, puis les polyanhydrides, sont actuellement les plus étudiés. Leur biodégradation, provoquée par des phénomènes d'hydrolyse, débute en présence d'eau.

La classe des polyesters aliphatiques comporte les poly-alpha-hydroxyacides et les poly-epsilo-caprolactones. Les poly-alpha-hydroxyacides sont constitués d'unités d'acide lactique et d'acide glycolique. Quand les deux types de monomères sont associés dans la même chaîne, on obtient un copolymère poly-lactide-co-glycolide (PLGA). Les produits de dégradation finale de ce copolymère sont l'eau et le CO_2. Les propriétés physicochimiques et la dégradation de ce copolymère dépendent de plusieurs paramètres comme le ratio des deux monomères, le poids moléculaire du polymère, l'index de polydispersité, etc. Le profil de dégradation (de quelques semaines à quelques années) peut donc être adapté à la situation clinique. Les PLGA sont utilisés en clinique humaine depuis 30 ans en sous-cutanée. Leur parfaite biocompatibilité avec le tissu cérébral a été établie (Menei et al., 1993). Le poly-epsilo-caprolactone qui se dégrade beaucoup plus lentement est aussi biocompatible avec le tissu nerveux (Menei et al., 1994a).

Les polyanhydrides sont d'utilisation plus récente en thérapeutique. Leur biodégradabilité peut également être modulée (Wu et al., 1994). La biocompatibilité avec le tissu cérébral du poly-1,3-bis (p-carboxyphenoxy) propane coacide sébacique, ou PCPP : SA, a été démontrée (Tamargo et al., 1989).

3.1. *Implants monolithiques*

Les premiers essais cliniques ont été réalisés au Japon dans les années 1980, avec des implants de silicone libérant du 5-FU (Oda et al., 1982) et avec des aiguilles d'acide polyméthyl-méthacrylique libérant de la mitomycine, de l'adriamycine, de l'ACNU ou du 5-FU (Kubo et al., 1994). Aucune conclusion ne peut être tirée de ces études en raison de l'hétérogénéité des tumeurs traitées.

Le Gliadel®, développé dans les années 1980, a été le premier à franchir toutes les phases de développement et d'évaluation précliniques pour être agréé aux États-Unis et en Europe, devenant pour l'instant, le seul « médicament » de sa catégorie.

C'est un disque de 14 mm × 1 mm, constitué de copolymère biodégradable, le poly-1,3-bis(p-carboxyphénoxy)propane-co-acide sébacique (ou PCPP : SA), libérant du BCNU au

moins pendant 21 jours. Le BCNU a été choisi, car c'est une molécule classiquement utilisée dans la prise en charge des gliomes malins, mais qui présente une forte toxicité systémique (hématologique et pulmonaire). Après un développement préclinique sur des modèles animaux (Brem et al., 1994 ; Grossman et al., 1992), les premiers essais cliniques ont débuté en 1989 chez des patients en récidive de tumeur gliale maligne. Les disques sont apposés sur les parois de la cavité après exérèse chirurgicale de la tumeur avec un maximum de huit implants. Après une étude de phase I/II (Brem et al., 1991), un essai multicentrique randomisé a été réalisé sur 222 patients en récidive de gliome malin, un bras recevant les implants contenant du BCNU (62 mg maximum), l'autre bras recevant des implants sans BCNU (Brem et al., 1995a). Une augmentation de la médiane de survie a été montrée chez les patients ayant reçu les implants contenant du BCNU et le Gliadel® a obtenu l'AMM dans les récidives de gliomes malins au décours de ce travail.

Le Gliadel® a ensuite été étudié dans les gliomes malins en première intention, la mise en place des disques était alors suivie d'une radiothérapie externe standard. Après une étude pilote et une phase I/II (Brem et al., 1995b ; Valtonen et al., 1997), une phase III multicentrique a été conduite, montrant là aussi un bénéfice de survie pour les patients traités (Westphal et al., 2003). L'AMM a été étendue aux gliomes malins nouvellement diagnostiqués, en association avec la radiothérapie.

D'autres études sont actuellement en cours, évaluant l'association du Gliadel® à d'autres thérapeutiques, en particulier les nouvelles chimiothérapies systémiques comme le témozolomide.

D'autres systèmes polymères libérant d'autres molécules ont été évalués chez l'animal en traitement intratumoral. Ces systèmes entraîneront peut-être un jour la mise en place d'essais cliniques. Parmi les molécules vectorisées les plus intéressantes, il faut citer les antiangiogéniques (Sipos et Brem, 2000), la camptothécine (Weingart et al., 1995), le cyclophosphamide (Judy et al., 1995), l'IUdR (Geze et al., 1999 ; Yuan et al., 2001), la tirapazamine (Yuan et al., 1999), le Taxol® (Walter et al., 1994) ou les interférons et interleukines pour l'immunothérapie (Wiranowska et al., 1998).

3.2. *Microparticules*

Plusieurs types de microparticules ont été testés chez l'animal, mais peu ont donné lieu à des essais cliniques. Nous avons développé dans le laboratoire des microsphères de PLGA, de 50 μm de diamètre, se dégradant en deux mois et libérant du 5-FU sur plus d'un mois (Boisdron-Celle et Benoit, 1995). La taille de ces microparticules permet de les implanter sous la forme d'une suspension, à main levée ou par stéréotaxie, sans léser le tissu cérébral (Menei et al., 1994b ; Veziers et al., 2001). Le 5-FU a été choisi, car il ne franchit pas la BHE, son activité antitumorale est augmentée par une administration prolongée, il n'est actif que sur les cellules en division, il est peu neurotoxique et enfin, il constitue un puissant radiosensibilisant.

Après des études précliniques (Menei et al., 1996), une première étude de phase I a été menée sur le glioblastome en première intention en 1995 (Menei et al., 1999). Après exérèse macroscopiquement complète de la tumeur, les microsphères ont été implantées dans les parois de la cavité de résection (dose totale de 32 et 132 mg de 5-FU). La radiothérapie externe standard (60 Gy fractionnés sur six semaines) a débuté dans les sept jours suivant l'implantation, pour profiter de l'effet radiosensibilisant. La tolérance a été satisfaisante, et les études pharmacocinétiques ont montré que le 5-FU était libéré localement pendant au moins un mois et que le passage dans la circulation systémique était faible et transitoire. Au vu de ces résultats, une étude multicentrique randomisée de phase IIb (microsphères associée à la radiothérapie versus radiothérapie seule) a été conduite, montrant un bénéfice sur la survie et une faible toxicité (Menei et al., 2005). Une phase III devrait voir le jour.

Parallèlement, une étude de phase I évaluant la tolérance de l'implantation stéréotaxique des microsphères libérant du 5-FU chez des patients dont la tumeur est considérée comme inopérable (profonde ou en zone fonctionnelle) a été concluante, confirmant la faisabilité de la technique (Menei et al., 2004).

Cette implantation stéréotaxique intratumorale de microsphères biodégradables peut également être utilisée pour administrer des molécules immunostimulantes comme l'IL18 (Lagarce et al., 2006 ; Koennings et al., 2006).

3.3. *Nanoparticules*

En raison de leurs propriétés physicochimiques et de leur taille pouvant varier classiquement de 1 à 100 nm, les nanosystèmes ou les nanoparticules peuvent interagir de façon unique avec les systèmes biologiques. Selon les orientations choisies, leurs conception, synthèse et caractérisation demeurent des travaux pionniers et les outils développés encore largement perfectibles. Toutefois, les études récentes ont déjà pu démontrer l'intérêt des nanotechnologies vis-à-vis des glioblastomes pour au moins deux missions locales essentielles, d'une part l'imagerie de ces tumeurs intracérébrales et, d'autre part, l'administration d'agents pharmacologiques. En ce qui concerne l'imagerie cérébrale, des nanosondes IRM couplées à la fluorescence dans le proche infrarouge ont révélé une affinité de capture par la tumeur elle-même (Veiseh et al., 2005 ; Trehin et al., 2006). Des nanoparticules superparamagnétiques de polyacrylamide et d'oxyde de fer ont également été développées et présentent une capacité de rétention au niveau tumoral (Moffat et al., 2003). Parallèlement, afin de renforcer encore les spécificités de ciblage et de reconnaissance des gliomes, des nanoparticules, sur lesquelles sont fixées des séquences RGD (arginine-glycine-acide aspartique) cycliques, peuvent reconnaître des intégrines surexprimées dans la tumeur (Montet et al., 2006). De tels nanosystèmes pourraient être utilisés dans le futur proche pour de l'imagerie en temps réel, lors des résections de tumeurs cérébrales, pour confrontation au diagnostic préopératoire avec résolution cellulaire.

En ce qui concerne l'administration d'agents cytotoxiques dans les glioblastomes, divers systèmes colloïdaux submicrométriques offrent l'opportunité d'améliorer la biodistribution et l'index thérapeutique des principes actifs qu'ils contiennent. Qu'il s'agisse de liposomes (Sells et al., 1987 ; Rahman et al., 1990 ; Cowens et al., 1993 ; Gabizon et al., 1994 ;

Storm et al., 1998), de nanosphères polymériques (Gref et al., 1994), de nanocapsules polymériques (Couvreur et al., 2002) ou de nanoparticules lipidiques (Muller et Keck, 2004), le devenir du principe actif n'est alors plus dépendant de ses propriétés intrinsèques, mais de celles de son vecteur. Parallèlement, il est protégé du reste de l'organisme jusqu'à sa libération, si possible à proximité de sa cible, pour plus d'efficacité, de spécificité et de sécurité biologique.

À cet égard, en utilisant une méthode d'émulsion par inversion de phase et en absence de l'utilisation de tout solvant organique, l'Inserm U646 a récemment mis au point et breveté des nanocapsules lipidiques (Heurtault et al., 2002). Ces nanocapsules lipidiques (LNC), dont la taille peut être ajustée de 20 à 100 nm avec une distribution monodisperse, sont capables de cibler le compartiment intracellulaire des cellules de gliomes et peuvent servir de réservoirs à partir desquels peut être libéré un agent anticancéreux tel que le paclitaxel qui agit sur les microtubules (Garcion et al., 2006). De manière intéressante, il a été mis en évidence une interaction entre les LNC et les pompes d'efflux, qui résulte en une inhibition de la MDR dans les cellules de gliome en culture et implantées chez l'animal (Garcion et al., 2006). Finalement, il a été établi que les LNC chargées en paclitaxel sont plus efficaces que le paclitaxel dans sa formulation classiquement disponible en clinique (Taxol®), augmentant la mort des cellules cancéreuses in vitro et réduisant l'expansion tumorale des gliomes implantées en sous-cutané in vivo (Garcion et al., 2006). Ces LNC semblent combiner les avantages spécifiques de deux autres nanosystèmes sensibilisateurs de cellules cancéreuses « multi-substances résistantes » : d'une part, ceux de micelles de copolymères blocs synthétiques qui présentent une toxicité in vitro jusqu'à 1000 fois supérieure à celle du principe actif non encapsulé et qui doit être reliée à leur rapide internalisation (Ramaswamy et al., 1997 ; Batrakova et al., 1999 ; Batrakova et al., 2003 ; Batrakova et al., 2004), et d'autre part ceux des nanoparticules de poly-alkyl cyanoacrylate (Hu et al., 1996 ; Soma et al., 2000 ; Vauthier et al., 2003), avec la possibilité de greffer des groupements de reconnaissance moléculaire comme des peptides ou des anticorps afin de renforcer un ciblage non seulement cellulaire, mais également subcellulaire. En ce sens, il convient de souligner que les caractéristiques de surface de ces nano-objets sont prépondérantes. Si des nanosphères de poly-hexadécylcyanoacrylate revêtues de polyéthylèneglycol sont capables de cibler des cellules de gliomes chez le rat sans améliorer l'évolution de la maladie lorsque chargées en doxorubicine (Brigger et al., 2004), des résultats opposés sont obtenus lorsque sont utilisées des nanoparticules chargées en doxorubicine et recouvertes de polysorbate (Steiniger et al., 2004).

Enfin, d'autres approches utilisant les nanotechnologies sont en cours d'étude, en combinaison avec la *convection enhanced delivery*, comme l'utilisation des nanoliposomes contenant du topotecan (inhibiteur de topoisomerase I) qui ont permis l'obtention d'effets bénéfiques importants alors que le produit seul restait inefficace (Saito et al., 2006). La thermothérapie utilisant des nanoparticules magnétiques est également un nouveau concept de thérapie tumorale locale basée sur le chauffage contrôlé de nanoparticules magnétiques préalablement injectées (Plotkin et al., 2006). Enfin, la thérapie par capture d'atome de boron au travers de composés boronisés sélectivement internalisés dans les cellules cancéreuses grâce à des nanosystèmes est une voie supplémentaire de recherche (Lu et al., 1997).

L'évolution de certains de ces nanosystèmes pourrait s'avérer être une étape essentielle dans le développement de protocole thérapeutique dirigé contre le glioblastome en clinique.

4. Conclusion

Les thérapies locales des gliomes, qui en sont encore à leur début, sont globalement mal comprises des oncologues et souvent décriées. Elles ont pourtant un avenir, aux cotés des thérapies systémiques. L'intérêt que porte l'industrie pharmaceutique à ces nouvelles stratégies est là pour le confirmer.

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✓ **Abstract n° 1**

- In situ therapy for glioma: from polymeric devices to nanotechnologies.

Vinchon-Petit S, Garcion E, Menei P.

Annual meeting of the International Brain Mapping & Intraoperative Surgical Planning Society – IBMISPS. September, 5-8, 2006. Clermont-Ferrand, France.

“In situ therapy for glioma: from polymeric devices to nanotechnologies”
Sandrine Vinchon-Petit (1,2), Emmanuel Garcion (1), Philippe Menei (1,3).
Annual meeting of the International Brain Mapping & Intraoperative Surgical Planning Society – IBMISPS. September, 5-8, 2006. Clermont-Ferrand, France.

1. Inserm U646, Angers, France.
2. Paul Papin Center, Angers, France.
3. Neurosurgery Department, University Hospital, Angers, France.

Chemotherapy for brain tumours is limited because of difficulty in achieving adequate exposure to the tumour without systemic toxicity. Moreover, gliomas develop a multidrug resistance (MDR). Methods for local sustained release of chemotherapeutic agents by their incorporation into biodegradable polymers have been performed. Biodegradable 1, 3-bis (2-chloroethyl)-1-nitrosourea (BCNU) wafers (Gliadel wafers) prolong survival in patients with recurrent and newly diagnosed malignant glioma. We have designed implantable biodegradable microspheres to provide the delivery of the radiosensitizer 5-fluorouracil (5-FU) after patients underwent surgical resection of malignant glioma or into inoperable brain tumors. But, the survival is not concurrently longer with these materials, the diffusion from the implants is poor and there is no MDR inhibition. By focusing on rat glioma, we have elucidated whether new lipid nanocapsules (LNC) may improve anticancer hydrophobic drug bioavailability while also overcoming multidrug resistance. Blank LNCs and LNCs loaded with the antineoplastic agent paclitaxel are formulated by an emulsion inversion phase process. We have demonstrated that convection-enhanced delivery (CED) improves their volume of distribution. We have evaluated the radiosensitizing effect of paclitaxel-loaded LNC (LNC-Px) managed by CED in 9L glioma. The rats have been randomized in 5 groups: untreated, radiotherapy alone (RTE), CED/paclitaxel+RTE, CED/LNC-Px+RTE and CED/ blank LNC+RTE. On day 60, 60% of the CED/LNC-Px+RTE group are still alive against 40% in the CED/ blank LNC+RTE group, 30% in the CED/paclitaxel+RTE and 20 % in the radiotherapy alone group. These data may represent an important step towards the development of new strategies against gliomas.

Key Words: Radiosensitization**,** Convection-Enhanced Delivery**,** Nanotechnologies**,** Paclitaxel, Glioma.

2. TRAVAUX PERSONNELS

2.1. Présentation du travail.

L'utilisation de la radiothérapie postopératoire est considérée depuis longtemps comme un standard dans la prise en charge du glioblastome, comme en témoignent 6 études randomisées publiées entre 1976 et 1991 (33). L'étude du SGSG (Scandinavian Glioblastoma Study Group), incluant 118 patients, confirme l'importance de la radiothérapie adjuvante avec une médiane de survie de 21 semaines en cas de chirurgie seule contre 44 semaines pour les patients irradiés (34).

Ces dernières années sont également marquées par l'amélioration de la survie grâce à la prescription d'une chimiothérapie face à une tumeur cérébrale de mauvais pronostic. Cairncross avait déjà révélé, dans les années 90, la chimiosensibilité de certaines tumeurs gliales de haut grade (35-38). Depuis, ce bénéfice a été évalué pour les glioblastomes dans divers essais randomisés. Le témozolomide, agent alkylant de 2ème génération, administré par voie orale, a clairement apporté un bénéfice sur la survie en concomitant puis en adjuvant à la radiothérapie, pour un total de 6 cycles, chez les patients porteurs d'un glioblastome (2). Suite aux résultats de cet essai international, le schéma de Stupp est devenu un standard thérapeutique dans la prise en charge des tumeurs gliales de grade IV (schéma 2).

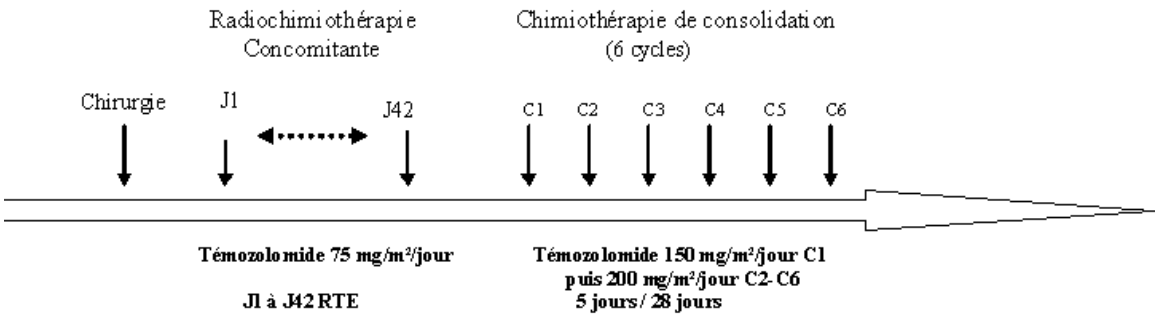

Schéma 2: Schéma thérapeutique (bras RTE+TMZ) évalué dans l'essai randomisé de phase III mené par l'EORTC et le NCIC (Schéma de STUPP) (7).

Ainsi, les études précliniques visant à démontrer l'efficacité de nouvelles thérapies dans la prise en charge du glioblastome nouvellement diagnostiqué ne peuvent s'affranchir de la radiothérapie. De plus, compte-tenu des résultats obtenus par Roger Stupp avec son schéma de radio-chimiothérapie concomitante par témozolomide et du fait des difficultés d'accès des tumeurs cérébrales par la chimiothérapie systémique, nous avons orienté nos travaux pour tenter d'améliorer la radiosensibilité des tumeurs gliales dans un modèle de gliome du rat par injection locale de chimiothérapie encapsulée. Nous avons utilisé différents outils récents allant des micro- aux nanotechnologies et mis à profit la méthode d'injection innovante qu'est la CED afin d'optimiser nos résultats.

2.2. Modèle de radiothérapie encéphalique dans le traitement du gliome 9L du rat.

a. Présentation du travail

Avant de débuter les études «thérapeutiques», nous avons revu la littérature afin de définir un protocole d'irradiation adapté au petit animal. Dans la littérature, il existe presque autant de schémas possibles que d'études. Quelques uns ont été sélectionnés et critiqués afin de nous aider à établir le protocole qui nous semblait le plus adapté. Les critères importants à définir étaient:

1. la dose par fraction
2. le nombre de fractions
3. l'étalement du traitement
4. le volume à irradier
5. l'appareil de traitement.

Nos exigences étaient:

1. *nous permettre d'étudier une chimiothérapie dont la finalité serait d'être utilisée en pratique clinique de manière concomitante.*

Le schéma devait donc être suffisamment étalé pour pouvoir mettre en évidence une éventuelle radiosensibilisation.

2. *permettre une certaine facilité dans les manipulations.*

Le schéma ne devait pas être toxique pour le rat, facile à mettre en place pour l'expérimentateur et reproductible afin d'exploiter au mieux les résultats des différents travaux.

✓ **Article n° 2**

- External irradiation models for intracranial 9L glioma studies.
Vinchon-Petit S, Jarnet D, Jadaud E, Feuvret L, Garcion E and Menei P. *article en correction (Journal of Experimental & Clinical Cancer Research).*

External irradiation models for intracranial 9L glioma studies

S. Vinchon-Petit[1, 2, *], D. Jarnet[2], E. Jadaud[2], L. Feuvret[3], E. Garcion[1] and P. Menei[1, 4.]

[1] INSERM, U646, Université d'Angers, Angers, F-49100, France.

[2] Department of radiation therapy, Centre Paul Papin, Angers, F-49100, France.

[3] Department of radiation therapy, Hôpital de la Pitié-Salpêtrière, Paris, F-75013, France.

[4] Department of Neurosurgery, CHU d'Angers, Angers Cedex 9, F-49933, France.

*To whom correspondence should be addressed:

Sandrine Vinchon-Petit, MD.

Department of Radiation Therapy, Centre Paul Papin 49933, Angers cedex 09, France.

Tel : (33) 241.35.29.16

Fax: (33) 241.35.25.35

E-mail: s.vinchon-petit@unimedia.fr

ABSTRACT

Purpose: Radiotherapy was shown to be effective on human glioma and consists of 30 fractions of 2 Gy each for 6–7 weeks in the tumor volume with margins. But in preclinical, many radiation schedules are used. The main purpose of this work was to review a part of the literature and to propose an external whole-brain irradiation (WBI) protocol for rat 9L glioma model.

Methods and Materials: 9L cells were implanted in the striatum of twenty 344-Fisher rats to induce a brain tumor. On day 8, animals were randomized in two groups: an untreated group and an irradiated group with three fractions of 6 Gy at day 8, 11 and 14. Survival and toxicities were studied.

Results: Irradiated rats had significantly a longer survival (p=0.01). Any death happened because of the treatment. Toxicities were increased during the radiation period for weight and hairs but there wasn't either serious toxicity morbidity nor mortality. Moreover, anomalies disappeared the week following the end of the therapeutic schedule.

Conclusions: Delivering 18 Gy in 3 fractions of 6 Gy with mild anaesthesia is safe, easy to reproduce and could be used to conduct preclinical studies on glioma rat model.

Keywords: Glioma, Radiotherapy, glioma rat model, 9L, Rat.

INTRODUCTION

Malignant glioma is the most frequently primitive brain tumor. Prognosis is extremely poor with current standards of treatment. Median of survival is less than fifteen months with a complete treatment associating surgery, radiotherapy (RT) and chemotherapy (1). Temozolomide, a novel alkylating agent, has shown modest activity against recurrent glioma. In combination with radiotherapy in newly diagnosed patients with glioblastoma, temozolomide significantly prolongs survival. Radiotherapy represents the mean part of the treatment (2-4). To be efficient enough and not toxic, RT consists of 30 fractions of 2 Gy each, administered Monday–Friday for 6–7 weeks (42 days) in the tumor volume with margins. The schedule is clearly defined (5). So, in preclinical studies about adjuvant therapies, radiation therapy can't be bypass. Previously, we used a fractionated irradiation delivering 36 grays in 9 fractions of 4 grays to treat C6 tumor bearing-rats (6). We found that brain radiotherapy for rat 9L-glioma- which is the most common preclinical model used- is not standardized. Moreover, the schedules described in literature are highly heterogeneous (Table 1) (6-13). To prove an interesting effect of a concomitant treatment, the radiation therapy protocol must be well defined. After a review of some studies published in literature, the aim of this work was to propose our brain irradiation model for rats, closer to clinical practice, safe for small animals and easy to reproduce in order to study concomitant treatments.

MATERIELS AND METHODS

All experiments have been conducted under good experimental practices. All animal handling have been carried out according to the European Community regulation and French Ministry of Agriculture regulations.

1. Animals

20 females Fischer-344 rats were used for this study (Charles River, Cleon, France). Ten weeks-old, they weighed 150 to 200 grammes. They were housed in groups of 4 in cages in conformity with the standards of the directives of the Union European and dealt with by the animal facilities of the Faculty of Medicine of Angers, establishment approved according to the law.

2. Tumor model

Rat 9L-glioma cells (European Collection of Concealment Culture, n° 94110705, Salisbury,U.K.) were cultivated in the "DMEM" medium ("Dulbecco's Modified Eagle's Medium", Biowhittaker, Verviers, Belgium) added with 10% of foetal calf serum (FBS, Biowhittaker) and of a mixture of antibiotics: penicillin (100 UI/ml), streptomycin (0.1 mg/ml) and amphothericin B (25 µg/ml) (ABS, Sigma, Saint Quentin Fallavier, France). Cells were maintained in a balanced wet atmosphere (37°C and CO_2 5%). Animals were anesthetized with an intraperitoneal injection of 0.75-1.5ml/kg of a solution containing 2/3 ketamine (100mg/ml) (Clorketam®, Vétoquinol, Lure, France) and 1/3 xylazine (20mg/ml) (Rompun®, Bayer, Puteaux, France). Rats were placed in a small-animal stereotaxic frame (Kopf Instruments, Phymep, France). After shaving and skin disinfection, a sagittal incision of 2 cm was made to expose the skull, followed by a burr hole 0.5 mm anterior and 3 mm lateral from the bregma using a small drill. After trypsinisation (trypsine/EDTA (Sigma)) and resuspension in "EMEM" ("Eagle's Minimum Essential Medium", Biowhittaker), 10µl of 10^3 9L-cells in suspension were implanted 5 mm deep in the right striatum (according to the Paxinos atlas) with a 10 µl -26G Hamilton

syringe (Harvard Apparatus, Ulis, France). After waiting 5 minutes, the needle was removed and the wound was sutured with absorbable surgical thread. Rats bearing 9L tumor were randomized either in the "untreated" group (group A) or in the group irradiated by a whole-brain irradiation (WBI) to a total dose of 18Gy (group B). The radiotherapy started at day 8 after the tumor cell implantation when the tumor size is 10-15 microlitres (14).

3. Radiotherapy protocol

Rats were irradiated using a 6-MV linear accelerator (Saturn 41 type, Varian Medical Systems, Salt Lake City, USA) in the radiotherapy department. During irradiations, mild anaesthesia by isoflurane (4.5% during 2 min then 2% for the treatment) + O_2 3L/min was performed. Oxygen masks were connected by four and four rats were placed in a reproducible way, in prone position on the linac couch with laser alignment. The WBI was delivered by one photons beam (6 MV-energy, DSP 100 and 4Gy/min). The field was 15 X 15 cm at source-axis distance, 100 cm. The isocenter was in the midline of the brain and the posterior limit of the field corresponded to the line passing by the posterior part of the 2 ears (Fig. 1).

A 15-mm thickness of equivalent tissue was laid on the rat's head in order to homogenize the dose received in brain. The dose distribution was studied by the physic's department. Eighteen grays, given in 3 fractions of 6 grays were delivered in 7 days in the isocenter corresponding to the tumor (Fig. 2). The brain was covered by the 95%-isodose. The irradiation started if there was any healing trouble (abscess, haematoma…) and if rat general state allowed it. After irradiation, animals were replaced in their cage. Control rats were also anesthetized according to the irradiation's schedule.

4. Animal observation

Rats were examined daily and staged for activity and well-being according to a classification developed in our animalery (data not published) (Table 2). Toxicities were noted. Rats were weighed weekly. Rats too weak to feed and to stand (corresponding to stage 2) were

sacrificed (atmosphere saturated with CO_2). The day of euthanasia was recorded and used in the survival analysis. All brains were removed and macroscopically examined when possible. It was noted if a tumor was found.

5. Statistics

Survivals were calculated from the day of the tumor implantation and presented as median and mean ± SE (Standard Error). Increase of life span (ILS) was calculated as follows: (Mean of Survival Max- Mean Survival Min)/ Mean of Survival Min x 100. Student t-test was performed to compare means of survival. SPSS® software was used for that purpose and tests were considered as significant with p values <0.05. Any rat surviving longer than 120 days was regarded as a 'long survivor'. The Kaplan-Meier method was used to plot animal survival.

RESULTS

1. Efficacy of the brain irradiation

The dosimetry planification is reported in figure 3. The 95%-isodose curve covered all the brain and 95% of the volume received 95% of the total dose. In the group A, two animals died during anaesthesia induction, before the tumor cells implantation. The brain was analyzed macroscopically in 12 animals (six in group A and six in group B). Degradation of brain in other animals, due to oedema, didn't allow analysis. For the 12 animals, a voluminous tumor was observed in their right striatum. On day 35, all rats from group A died. Mean of survival of this untreated group was 28.1 days ± 1.3. For group B, mean of survival was 59.9 days ± 8.2 (Table 3). The rate of long survivors in this group was 20% (2 rats / 10). The macroscopic examination of their brain was normal with no sign of tumor or injection trail. So, we didn't perform microscopic analysis. Rats treated with 18 Gy showed an increased mean survival time of 113% when compared to controls. The result in survival time was significantly different compared to the control group ($p= 0.01$) (Fig. 4).

2. Schedule toxicity

No rat, in any group, developed evident behavioural anomalies before approximately four days preceding death. Rats were either sacrificed at stage 2 to avoid suffering or died spontaneously during the night (n=8). There were no issues with wound healing following the procedure. All rats in group B developed complete but reversible (WHO grade III) alopecia on the surgical site during radiation therapy. It was reversible during the 21 days following the last day of irradiation. There was no healing problem at the skull incision. During the radiation therapy (d8-d14), the general behaviour was preserved with no feeding trouble but the weight increase wasn't as important as the one observed for rats in group A. For group A, values of the weight noted during the first 14 days were the same that those observed for twelve weeks old-

rats. The mean increase of weight for the “untreated” group was of 7.69% between d8 and d20 versus 2.47% for the irradiated group. The difference was significant (p= 0.01). Previously, mean time of survival of the untreated group being 27.5 days, loss of weight would have been noted for a significant number of rats in connection with neurological degradation related to the tumor evolution. So, for group A, values of the weight increase after day 20 resulted from an extrapolation starting from the weight increase noted during the first 14 days. Weight evolution became not significantly different one week after the end of radiation therapy (p=0.25) with an increase of weight estimated at 3.79% for group A and an increase of 6% for the group B. The evolution of the weight graph for 2 groups during the observation is reported on figure 5. We didn’t find any other clinical disorder due to irradiation.

DISCUSSION

Even though the one-fraction irradiation was well tolerated in literature, we decided to use a fractionated protocol to irradiate rats. Using a fractionated radiotherapy is closer to clinical practice and more adapted for a preclinical study, especially for a daily concomitant chemotherapy as Stupp defined it for human gliomas (1). In literature, the number of fractions goes from 5 to 20 (Table 1) (6, 8, 9 and 12). A limit for a fractionated radiotherapy with small animals could be the reproducibility concerning the position. With three fractions, it is easier to be reproducible and to assure a good quality of treatment compared to more-fractionated protocols. Rats have to be anesthetized and especially if one hemi-brain irradiation is required. However, most of drugs used for anaesthesia have effects on blood brain pressure, which is already high when a brain tumor grows or are known to be radioprotectant for the normal brain parenchyma. Ketamine, which is usually used for anaesthesia of rodents, induces a general increase in cerebral blood flow at anaesthetic concentrations (15). Some authors published that Pentobarbital protects against radiation-induced damage to normal rat brain. Even though there isn't conclusive evidence for either radioprotection or significant improvement of radiotherapeutic efficacy, in 9L rat brain tumor model, pentobarbital could induce the selective protection of normal brain (11, 13). In our model, anaesthesia with isoflurane is easy to use every three days, is well tolerated by rats with a complete and immediate recovery after irradiation and doesn't interfere with normal or brain tumor cells. Some use Plexiglas stereotactic frames for rat positioning and treat just one hemibrain. Previously, in our laboratory, we used a fractionated radiotherapy in one hemibrain (6). We found that the hemibrain irradiation isn't good enough as observed in figure 6. The volume of interest is better covered when all the brain is treated

because of the small size of a rat brain. The Histogram-Dose Volume (HDV) obtained for the two modalities of treatment are represented in figure 7.

The dose to apply per fraction is uncertain to treat a rat brain glioma. Our protocol was selected based on the linear-quadratic formula with α/β of 10 for the tumor and α/β of 3 for the normal tissue. The effective biological dose for the normal tissue is 32 grays and 27 grays for the tumor. These doses correspond to the dose received in clinical practice for a whole brain irradiation. We noted a mild and transitory toxicity which was quickly reversible after treatment. 9L cells are classified as a radioresistant cell line especially compared to other rodent glioma cell lines (16). Bencokova described a surviving fraction at 2 Gy (SF2) of 71.9% for 9L cells against 53.0 and 41.4% for C6 and F98 cell lines respectively (16). According to this, the dose to deliver by fraction must be higher than 2 grays. The dose per fraction in literature ranges from 2 to 40 grays (Table 1). For Kimler, the survival improvement is limited by the development of normal tissue toxicity at high doses (11). Kim observed that 35 Gy produced severe optic neuropathy (17). In his study, he tested a single high dose of radiation (ranging from 20 to 45 grays) with radiosurgery in a limited volume. With all these data, because the treatment must be efficient but not toxic, we decided arbitrarily to deliver fractions of 6 grays. Previously (18), we investigated a radiation therapy schema in 3 fractionated doses of 6 grays a week *in vitro* on 9L cell lines without and with concomitant chemotherapy. The results showed that cell death was most important as the number of fractions increased from 1 to 3 and the profit was better for the schemas associated with chemotherapy. For all the conditions tested, the main benefit in cell death was obtained after the first fraction (60-75% cell death), and was slightly reduced after the second and the third fraction. But the most important observation is that, the synergistic effect between chemotherapy (CT) and RT was most visible after the third fraction as cell death increased from 5.3% to 38.2% for the cells treated with RT alone versus CT + RT respectively. After the third fraction, the cell percentage still alive was mainly due to the radioresistance mechanism described above.

Two rats lived more than 120 days. They were sacrificed and their brain was removed. We couldn't decide in favour of a technical problem during the tumor cells implantation or a complete response after irradiation.

This work doesn't answer to a crucial question of therapy but it was made before studies we conducted in our laboratory to study the efficiency of local chemotherapy concomitant to radiation therapy in 9L glioma (19). Another study proved the reproducibility of the model because we obtained the same results in the radiation group compared to the untreated group (18). This radiation therapy protocol induces a strong tumor debulking and facilitates the chemotherapy treatment.

There is a paucity of experimental data in literature on rat radiobiology. Different energies are used. Some worked with a dedicated irradiator for small animals in their laboratory. This type of irradiator uses ^{137}Cesium or ^{60}Cobalt source and delivers gamma-rays (9, 20, 21 and 22). As Lamproglou, even though his work was about normal brain (12), we decided to treat our rats with linear accelerator used in clinical. Animal irradiation could be difficult to manage because of the availability of accelerators but the advantage is to deliver the same energy compared to clinical practice. There would be others advantages to use a nonradioactive source x-ray-producing irradiator such as avoiding the increasing number of radioprotection controls as well as the potential source hazard, disposal and replacement even though the attempted efficacy is the same whatever the radiation source chosen .

CONCLUSION

Many models of radiation therapy for rat glioma are available, with different schedules. We describe a reproducible paradigm of fractionated radiotherapy for rat bearing a brain tumor, with a good compromise between feasibility and adaptation to radiosensitization studies.

AKNOWLEDGMENTS

The authors would like to thank Pierre Legras and Jerome Roux (Service Commun d'Animalerie Hospitalo-Universitaire, Angers, France) for skillful technical support with animals and the Radiotherapy Department of Paul Papin Center for technical help. This work was supported by "La Fondation pour la Recherche Médicale".

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LEGENDS

Fig. 1: Radiation therapy position.

Fig. 2: Therapeutic schedule.

Fig. 3: Dose distribution in the whole rat brain.

Fig. 4: Survival times (days) after tumor implantation have been plotted for “untreated animals” (Group A) and “WBI (3 fractions of 6 Gy)” animals (group B).

Fig. 5: Evolution of the weight median depending on time of observation according to the group.

Fig. 6: Dose distribution in one hemibrain (A) and in the whole rat brain (B).

Fig. 7: Histogram-Dose Volume according to the treatment received. Green: hemibrain irradiation. Red: whole brain irradiation.

Table 1: Studies using radiation therapy rat model in combination with anticancer therapeutic agents.

Table 2: Rats’ staging.

Table 3: Descriptive and statistical data from the survival study depending on groups of treatment.

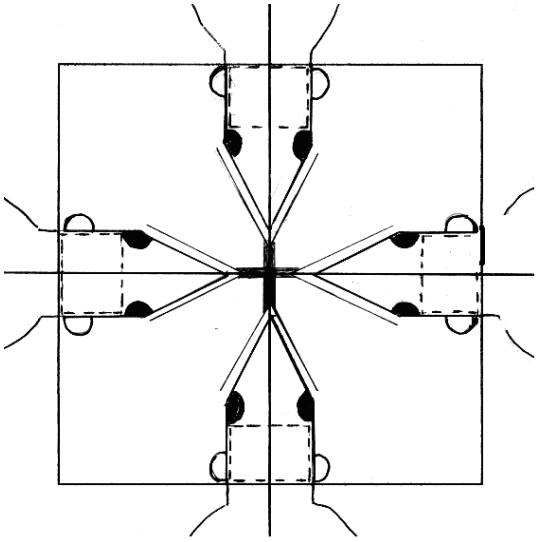


Fig. 1: Radiation therapy position.

Rats were placed in a reproducible way, in prone position, with laser alignment with gas mask for anaesthesia with isoflurane. The whole-brain irradiation was delivered by one photons beam (6 MV-energy, DSP 100 and 4 Gy/min). Radiation field was 15 X 15 cm large. The posterior limit of the field corresponded to the line passing by the posterior part of the 2 ears. An equivalent tissue of 1.5 cm was laid on the rat head in order to improve the dose distribution in brain.

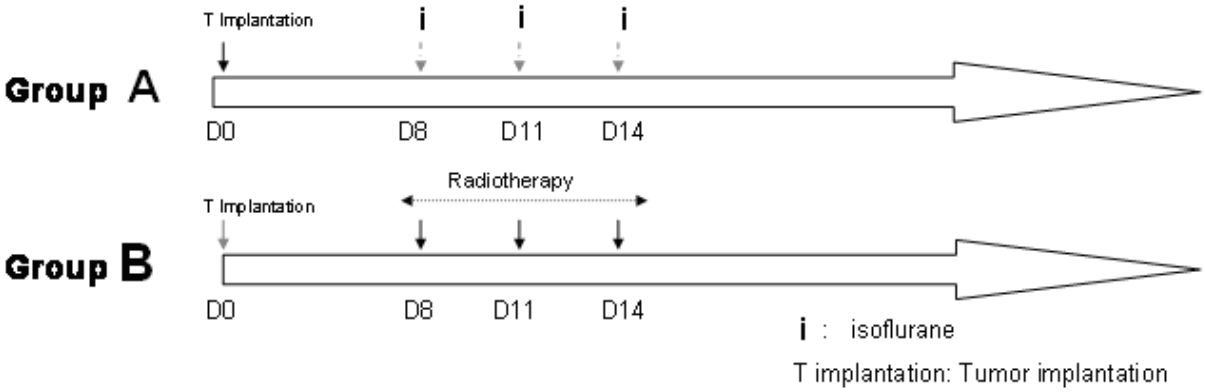


Fig. 2: Therapeutic schedule. On Day 0, 9L-tumor cells were implanted in the rat brain with intraperitoneal anaesthesia. Whole Brain Irradiation (WBI) began on Day 8 followed by 2 others fractions (6 Gy/ fraction) on Day 11 and 14 with isoflurane. Group A was untreated (control group). Group B was the irradiated group.

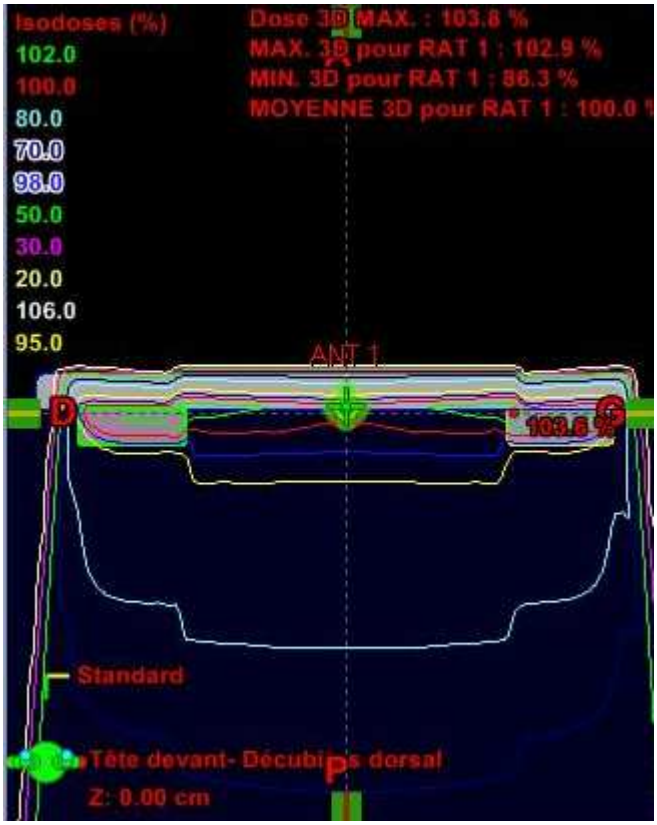


Fig. 3: Sagittal view of the dose distribution in the whole rat brain. On D and G are represented two of the four rat brains treated. The 95%-isodose curve is yellow and covers the whole brains. A 15-mm thickness of equivalent tissue was laid on the rats head (blue). The mean dose received is 100% of the dose delivered.

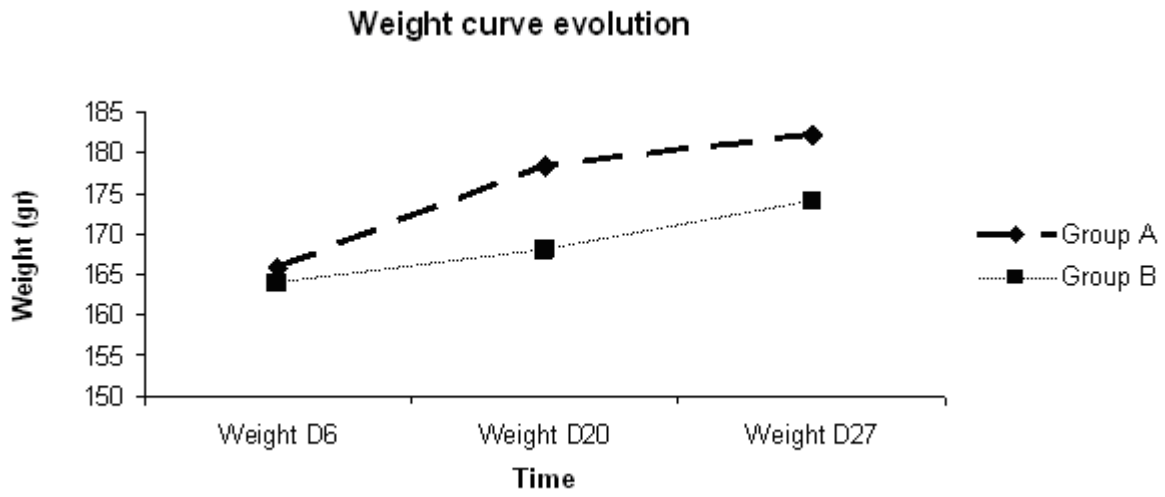


Fig. 4: Evolution of the weight median depending on time of observation according to the group. Group A corresponded to the "untreated" group and group B to the "WBI" group.

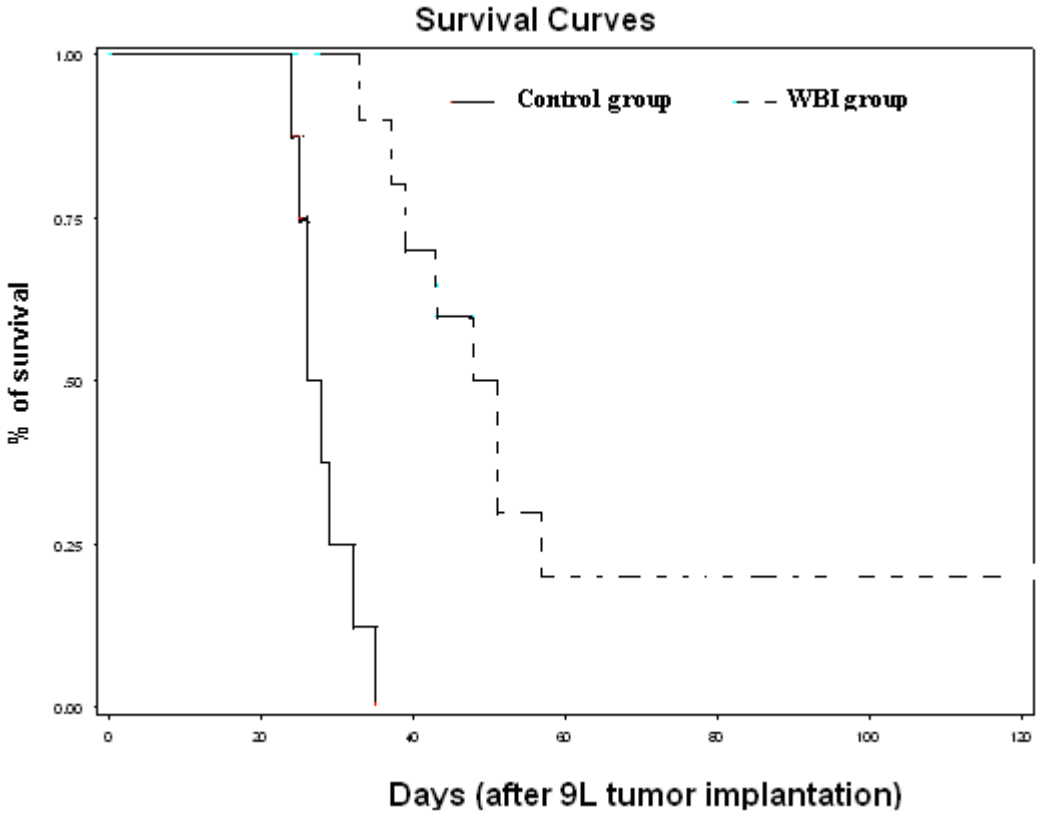
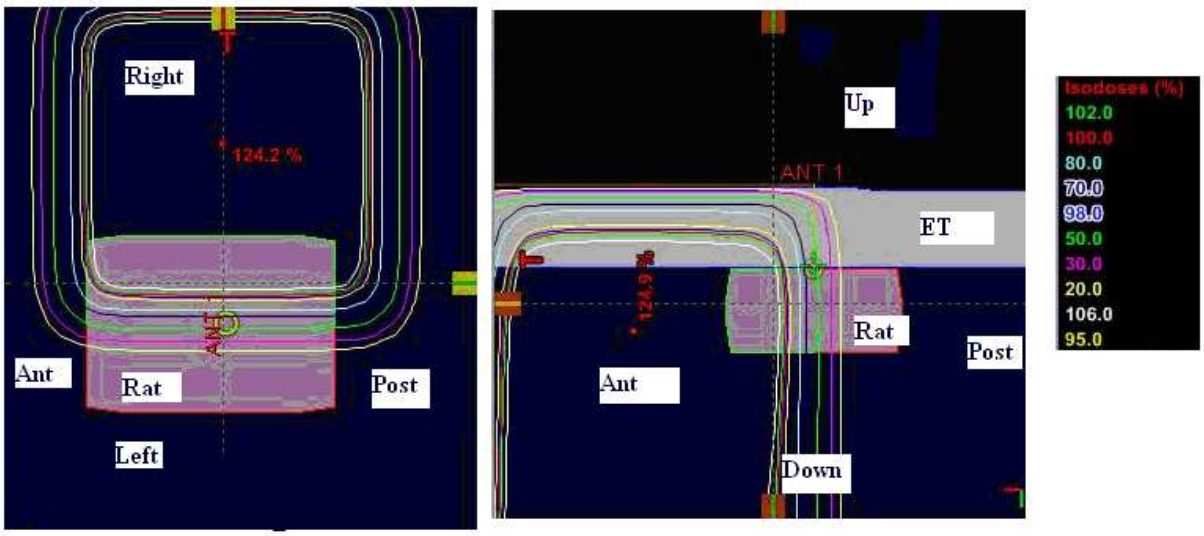
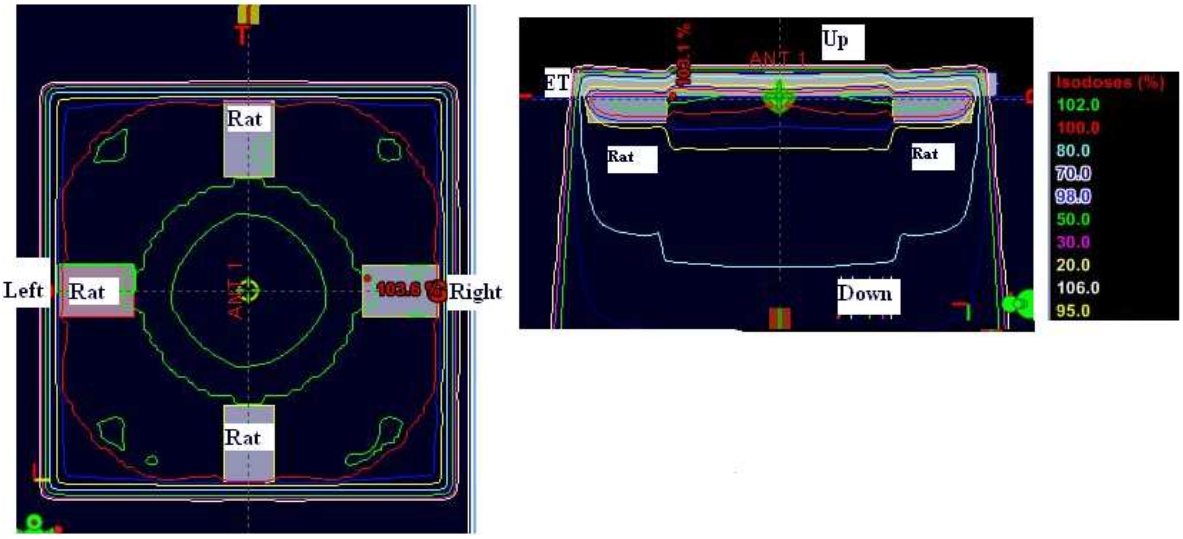


Fig. 5: Survival times (days) after tumor implantation have been plotted for “untreated animals” (Group A) and “WBI (3 fractions of 6 Gy)” animals (group B). Fractions were delivered on days 8, 11 and 14 after tumor implantation. (p=0.01).



A : Hemibrain irradiation
(ET= Equivalent Tissue; yellow: 95%-isodose curve)



B : Whole brain irradiation
(ET=Equivalent tissue; yellow: 95%-isodose curve)

Fig. 6: Dose distribution in one hemibrain (A) and in the whole rat brain (B).

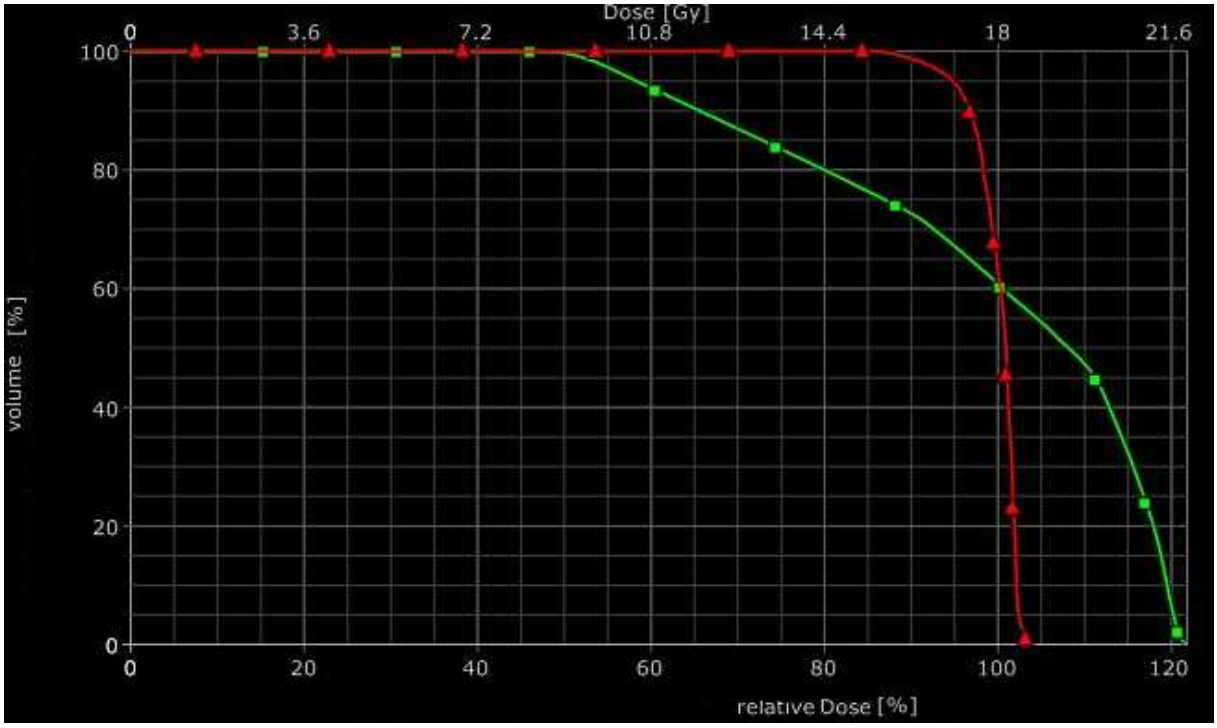


Fig. 7: Histogram-Dose Volume according to the treatment received. Green: hemibrain irradiation. Red: whole brain irradiation.

TABLES

Studies	*Target*	*Tumor Cell line*	*Total dose*	*Number of fractions*	*Survival*
Roullin VG (6)	HB	C6	36 Gy	9	Complete response : 8%
Graf MR (7)	WB	T9	15 Gy	1	35 days (median)
Kimler BF (8)	WB	9L	20 Gy	1	S
			30 Gy	5	S
Kimler BF (9)	WB	9L	40-70 Gy	10-20	S
Kimler BF (10)	WB	9L	16 Gy	1	38.5 days (mean)
Kimler BF (11)	WB	9L	16 Gy	1	S
			24 Gy	1	S
			32 Gy	1	S
			40 Gy	1	S
Lamproglou I (12)	WB	-	30 Gy	10	-
Olson JJ (13)	WB	9L	30 Gy	1	29.7 days (mean)

WB: Whole brain / HB: Hemibrain / S: Significant

Table 1: Studies using radiation therapy rat model in combination with anticancer therapeutic agents. Radiation therapy after intracranial tumor inoculation produced a significant improvement.
NB: Lamproglou worked on normal rat brains.

	Stage 5	Stage 4	Stage 3	Stage 2	Stage 1
Motility	Normal	Normal	+ but not spontaneous	Reduced	-
Stature	Normal	Stooped +	Stooped ++	Stooped +++	Dying
Piloerection	-	+/-	+++	+++	+++
Eyes	Sharp	Redness+	Redness ++	Eye secretions	Closed

Table 2: Rats staging (data not published).

Rats were examined daily and staged according to their motility, stature, hairs and eyes. Rats were weighed weekly. Rats too weak to feed and to stand (corresponding to stage 2) were sacrificed.

GROUPS	*Median of survival (days)*	*Mean time of survival (days) ±SE*	*Mean ILS (%)*	*Long term survivors*	*Maximal time of survival (days)*
Group A « untreated » (n=8)	27	28.1±1.3	-	0	35
Group B « WBI » (n=10)	49.5	59.9±8.2	113	2	120

Table 3: Descriptive and statistical data from the survival study depending on groups of treatment. Group A: group control "untreated"; group B: "Whole Brain Irradiation (WBI)" with 18 Gy (3 x 6 Gy).
Increase in life span (ILS) is calculated in comparison to the control group (%). SE means "standard error".

✓ **Abstract n°2**

- External irradiation models for intracranial 9L glioma studies.
Vinchon-Petit S, Jarnet D, Jadaud E, Feuvret L, Garcion E and Menei P.
15th Annual Scientific Meeting of the Society of Neuro-Oncology. November 18-21, 2010. Montreal. Canada.

ABSTRACT

Purpose: Radiotherapy was shown to be effective on human glioma and consists of 30 fractions of 2 Gy each for 6–7 weeks in the tumor volume with margins. But in preclinical, many radiation schedules are used. The main purpose of this work was to review a part of the literature and to propose an external radiotherapy protocol for rat 9L glioma model.

Methods and Materials: 9L cells were implanted in the striatum of twenty 344-Fisher rats to induce a brain tumor. On day 8, animals were randomized in two groups: an untreated group and an irradiated group with three fractions of 6 Gy at day 8, 11 and 14. Survival and toxicities were studied.

Results: Irradiated rats had significantly a longer survival (p=0.01). Any death happened because of the treatment. Toxicities were increased during the radiation period for weight and hairs but there wasn't either serious toxicity morbidity nor mortality. Moreover, anomalies disappeared the week following the end of the therapeutic schedule.

Conclusions: Delivering 18 Gy in 3 fractions of 6 Gy with mild anaesthesia is safe, easy to reproduce and could be used to conduct preclinical studies on glioma rat model.

Keywords: Glioma, Radiotherapy, glioma rat model, 9L, Rat.

SNO
The Society for NeuroOncology

The 15th Annual Scientific Meeting of the Society for Neuro-Oncology

November 18-21, 2010 • Montreal, Canada

Dear Dr. Vinchon-Petit,

Congratulations! Your abstract, 137, **External irradiation models for intracranial 9L glioma studies** has been accepted for the 2010 SNO meeting to be held in Montreal November 18-21. We expect to have allocations between platform and poster presentations complete within the next month. However, please note that, if you wish to withdraw your abstract, you must notify us before July 30 to ensure that your abstract will not be published in the SNO meeting abstract issue of the journal *Neuro-Oncology*. The presenting author should confirm intention to present research contained in the abstract, by fax to 832-201-8129, or email to jan@soc-neuro-onc.org.

If you have any questions regarding this process, please contact Jan Esenwein jan@soc-neuro-onc.org.

Sincerely,
Kenneth D Aldape
Program Chair

Society for Neuro-Oncology
4617 Birch Street
Bellaire, TX 77401-5509

b. Discussion

Notre travail ne répond pas à une question cruciale d'ordre thérapeutique. Il a favorisé la mise en place d'un schéma d'irradiation bien défini et a permis de mener à bien plusieurs études où l'objectif principal était de tester une molécule à visée radiosensibilisante. A chaque fois, les résultats obtenus dans les bras «radio-chimiothérapie concomitante» ont pu être comparés à ceux obtenus dans le bras «radiothérapie». Ce qui n'est pas systématiquement le cas dans ce genre d'études, même dans celles publiées parfois dans de grandes revues (39-40). Un point fort de ce protocole est sa reproductibilité que nous avons pu vérifier à l'occasion de plusieurs travaux (articles n°3, 4, 5 et 6). Cette qualité de traitement est probablement liée au choix de l'anesthésique. L'isoflurane est une drogue anesthésiante qui induit une sédation rapide et rapidement réversible à l'arrêt de l'exposition. De plus, elle ne majore pas la pression intracrânienne déjà augmentée par la tumeur cérébrale contrairement à d'autres anesthésiques fréquemment utilisés comme la kétamine (41). L'anesthésie des rats est donc facilement réalisable et permet un repositionnement fiable d'une séance à l'autre. Certains auteurs utilisent des boîtes de contention en plexiglas afin de s'affranchir de toute anesthésie. Il nous a semblait que cette technique était moins précise car le rat pouvait quand même bouger pendant la séance. Or un déplacement même minime soit-il peut avoir une incidence directe sur la qualité du traitement compte-tenu des petits volumes irradiés. L'autre point important de ce travail est que nous avons choisi de traiter les rats avec un accélérateur linéaire de type CLINAC et non avec un projecteur de source radioactive portatif dédié au petit animal. Le type d'énergie utilisé est donc le même que celui utilisé en clinique avec des énergies de rendement bien connu. Cette technique assure également une meilleure radioprotection des expérimentateurs.

Cependant, ce travail n'est qu'une étude préliminaire et d'autres expérimentations sont nécessaires afin de renforcer l'intérêt scientifique de notre protocole. Nous avons réalisé quelques tests d'irradiation *in vitro* sur des cultures cellulaires afin d'observer leur évolution en fonction de la dose délivrée. Nous pourrions reprendre ce travail *in vivo* sur plusieurs groupes de

rats. Nous pourrions également comparer des schémas avec des fractionnements variables. Nous avons également obtenu 20% de longs survivants après avoir traité nos rats. Ce constat a été rapporté dans d'autres études où des tumeurs 9L ont également été traitées. Les cellules 9L sont une lignée de cellules plutôt radiosensibles (42). Par contre, leur avantage est de former des tumeurs facilement reproductibles et ainsi mesurables car bien délimitées. Les cellules F98 sont à l'origine de tumeurs cérébrales invasives donc plus représentatives des tumeurs gliales humaines (43). Mais ceci les rend difficilement comparables. Il n'existe pas de modèle parfait de tumeur cérébrale chez le rat. Ainsi, dans ce type de pathologie pour lequel le pronostic est mauvais, les essais cliniques de phase I sont d'une importance fondamentale.

Une fois cette étape préliminaire réalisée, nous avons donc mis en place notre travail qui visait à améliorer la radiosensibilité des gliomes 9L et qui avait pour objectifs en termes de thérapie locale:

1. étudier la biocompatibilité et l'efficacité des microsphères chargées en doxorubicine ou irinotécan, synthétisées par Biocompatibles© et validées actuellement en pratique clinique dans la prise en charge des tumeurs hépatiques primitives ou secondaires.
2. utiliser la méthode de CED, inconnue du laboratoire pour l'injection locale intracérébrale de nanotechnologies.
3. tester pour la première fois *in vivo* le pouvoir radiosensibilisant des LNC de paclitaxel mises au point dans le laboratoire.

2.3. Utilisation des DC Beads® à visée radiosensibilisante dans le traitement local des gliomes 9L du rat.

a. Présentation du travail

- ✓ Les “DC Beads®”

Les microsphères testées dans ce travail peuvent être chargées de doxorubicine qui appartient à la famille des inhibiteurs de la topo-isomérase II ou d’irinotécan, inhibiteur de la topoisomérase I (Figure 7).

Les microsphères qu’elles soient blanches, chargées de doxorubicine ou d’irinotécan, sont produites par Biocompatibles UK Ltd (Farnham, UK) à partir d’un hydrogel d’alcool polyvinyle (PVA), biocompatible, qui a été modifié par des groupes sulphonate qui permettent une charge et une libération contrôlées des agents cytotoxiques (Drug-eluting beads) (Photo 6). Après une administration intra-artérielle, les microsphères stoppent le flux sanguin et libèrent localement, directement au sein de la tumeur, une dose contrôlée de cytotoxique (44-46). Les microsphères blanches ont reçu l’appellation “DC Bead®” en 2003. Les “DC Beads®” ont obtenues l’AMM dans le traitement des tumeurs malignes hypervascularisées. Lorsqu’elles sont chargées de doxorubicine, elles sont indiquées dans le traitement des tumeurs hépatiques qu’elles soient primitives (carcinome hépatocellulaire (CHC), cholangiocarcinomes, cancers neuroendocrines) ou secondaires (métastases hépatiques) (47-53). Les “DC Beads®” chargées d’irinotécan sont en cours d’investigation pour le traitement de patients atteints de cancer colorectal métastatique (54-57). Elles viennent également d’être testées dans la prise en charge des tumeurs pancréatiques ou de la carcinose péritonéale (58, 59).

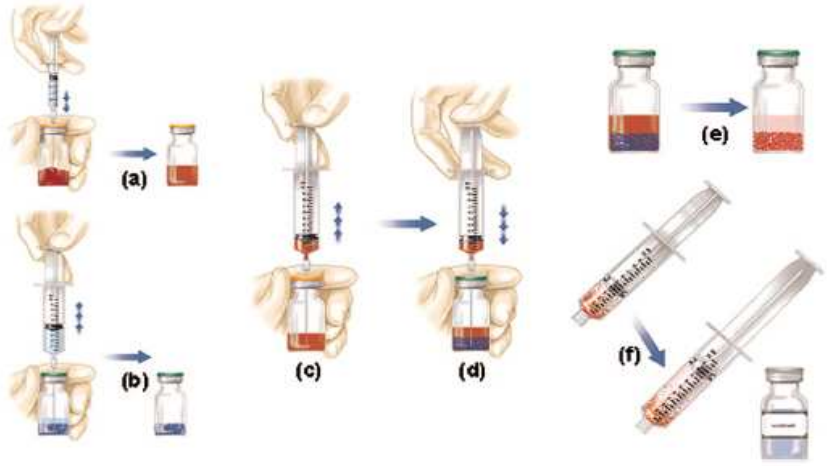


Figure 7: Procédé de chargement des microsphères en doxorubicine.

(a): une solution de doxorubicine est obtenue par ajout d'eau stérile à de la poudre de doxorubicine hypochloride.

(b): la solution saline est retirée par aspiration du flacon contenant les microsphères blanches.

(c) et (d): la solution de doxorubicine réhydratée est mélangée aux microsphères blanches.

(e): un temps de repos est observé pour le chargement des microsphères en doxorucibine. Il est fonction de la taille des microsphères et de la concentration finale désirée des microsphères en doxorubicine.

(f): la solution de microsphères chargées en doxorubicine est aspirée dans une seringue, prêtes à l'emploi.

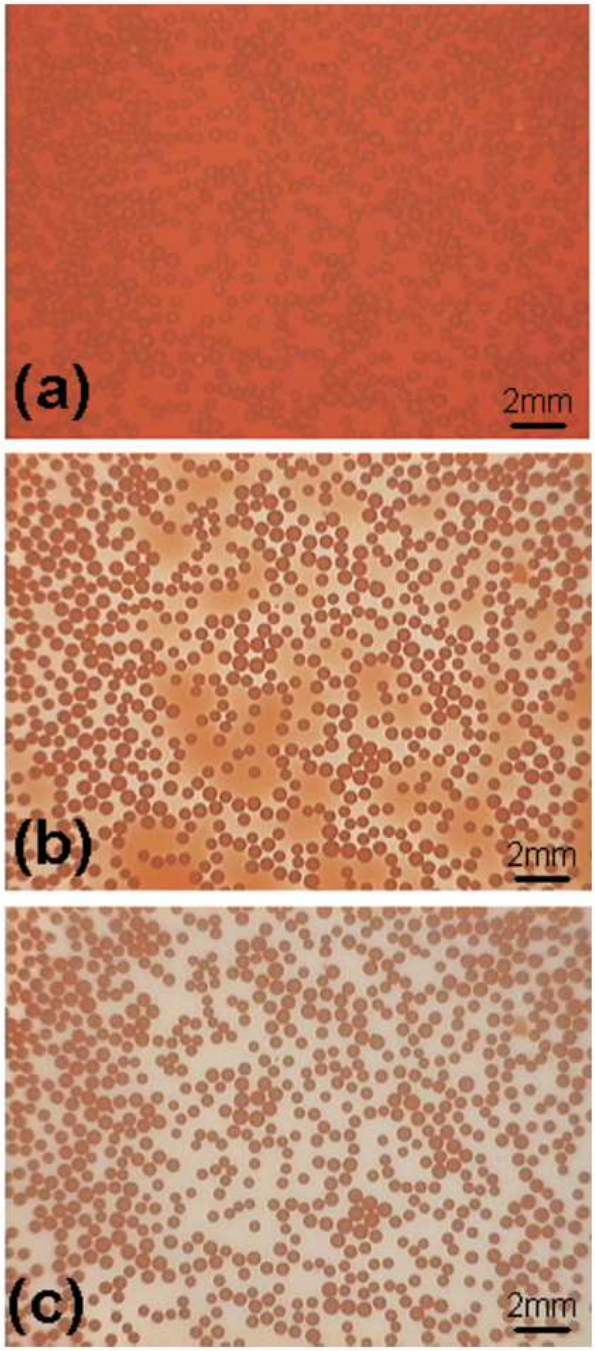


Photo 6: Exemple de chargement des microsphères (300-500 µm) en doxorubicine à 1 minute (a), 10 minutes (b) et 20 minutes (c).

✓ Doxorubicine et irinotécan dans la prise en charge des tumeurs gliales

Aujourd'hui, deux traitements ont obtenu l'AMM dans la prise en charge des patients atteints de glioblastome nouvellement diagnostiqué: l'association radio-chimiothérapie concomitante par témozolomide selon le schéma proposé par Roger Stupp et le Gliadel® apposé dans la cavité de résection chirurgicale en per-opératoire (2, 15). Il n'y a pas de standard concernant la combinaison de ces deux traitements en 1ère ligne chez les patients atteints de glioblastomes et plusieurs études sont en cours.

Chez les patients atteints de glioblastome récidivant, le pronostic est mauvais, avec une médiane de survie de 3 à 6 mois. Il n'existe pas de prise en charge standard mais lorsqu'une seconde intervention chirurgicale est possible, du Gliadel® peut être implanté. La combinaison bevacizumab + irinotécan a été étudiée dans plusieurs essais de phase I. Ce traitement constitue une nouvelle option actuellement très prescrite en France, car il existe peu d'alternatives (60). Les résultats montrent une amélioration de la survie sans récidive même si la survie globale n'est pas modifiée. La tolérance de l'association est essentiellement marquée par la survenue d'hypertension artérielle qui peut être contrôlée par un traitement symptomatique. La survenue d'une protéinurie doit être surveillée. L'irinotécan avait déjà démontré une certaine efficacité contre les tumeurs gliales (61-63). Les résultats obtenus dans les études l'associant avec le bévacizumab et ceux observés en pratique clinique quotidienne renforce cette hypothèse.

Il en est de même pour la doxorubicine qui est une molécule connue de longue date maintenant. La doxorubicine appartient à la famille des anthracyclines (64). Elle bloque la synthèse de l'ADN et de l'ARN en inhibant la topo-isomérase II. Elle s'est révélée efficace contre de nombreuses tumeurs malignes. Elle est actuellement prescrite dans les protocoles traitant les leucémies lymphoïdes aigües, les lymphomes, le myélome multiple, les sarcomes, les mésothéliomes, les tumeurs germinales de l'ovaire ou du testicule, les cancers du sein et les neuroblastomes (65). Les essais cliniques utilisant la doxorubicine par voie systémique ont démontré une efficacité limitée de cette molécule dans le traitement des gliomes. De très hautes

doses de doxorubicine doivent être utilisées par voie IV si l'on veut obtenir un bénéfice clinique suffisant. Or ces doses se révèlent être très toxiques (66, 67). Ce manque d'efficacité peut être expliqué par la présence de la BHE et des pompes à efflux qui ne permettent pas l'obtention d'un index thérapeutique suffisant au sein de la tumeur. La faible liposolubilité et le haut poids moléculaire de la doxorubicine empêchent son passage au travers de la BHE (66-68). Malheureusement, la doxorubicine s'était révélée très prometteuse *in vitro* contre les lignées cellulaires gliales (69-71). Des études précliniques sur des modèles animaux étaient même encourageantes (72-79). Il existe une étude clinique publiée récemment dans laquelle les résultats étaient en faveur de l'utilisation de la doxorubicine par l'intermédiaire d'un réservoir d'Ommaya (80). Cinquante pour cent des patients ont présenté une réponse radiologique objective. Ils n'ont pas développé de toxicité neurologique ou générale. Ainsi, l'administration intracérébrale de doxorubicine semble bien tolérée et pourrait être efficace dans la prise en charge des glioblastomes.

- ✓ <u>Rationnel du travail</u>

Initialement, le travail nous a été proposé par Biocompatibles UK Ltd (Farnham, UK). Riche de leur expérience dans les tumeurs hépatiques, ils ont souhaité étudier l'impact de l'utilisation des microsphères de doxorubicine et d'irinotécan dans la prise en charge des tumeurs gliales de haut grade. Leur demande répondait à notre souhait de poursuivre nos investigations dans le domaine de la radiosensibilisation par chimiothérapie locale encapsulée. Les microsphères de Biocompatibles© présentent plusieurs avantages:

1. Elles sont chargées en doxorubicine et irinotécan dont l'efficacité a déjà été prouvée dans la prise en charge des tumeurs gliales comme nous l'avons décrit précédemment.

De plus, ces molécules de chimiothérapie possèdent également des propriétés radiosensiblisantes. Elles constituaient donc de bonnes candidates pour poursuivre nos travaux.

2. Avant l'obtention de leur AMM, les microsphères ont fait l'objet d'études précliniques in vitro et in vivo puis d'études cliniques de phase I-II et III. Leur profil d'action et leur tolérance sont donc bien connus.

3. Elles existent en différentes tailles variant de 100 à 700 µm et en différentes concentrations de principe actif. Leur application pouvait être transposée au petit animal (Figure 8).

Doxorubicin Sequestration Rates of Varying Sizes of DC Bead

Bead Size Range (µm)	Doxorubicin Loading (mg/mL Beads)	Time to ≥99% Loading (min)
100–300	25	20
300–500	25	60
500–700	25	90
700–900	25	120
500–700	5	20
500–700	10	45
500–700	37.5	360
500–700	45	1440

Figure 8: Exemples de microsphères chargées en doxorubicine disponibles sur le marché variant en taille et en concentration.

Néanmoins, malgré ses éléments, leur utilisation dans le cadre des tumeurs cérébrales restait était innovante. Les résultats publiés précédemment nous ont uniquement permis de nous affranchir des étapes de caractérisation *in vitro*.

- ✓ Etapes préliminaires à l'utilisation intracérébrale des microsphères chez le rat.

Nous avons dû tester différentes méthodes de préparation et d'injection des microsphères avant d'initier les études de biocompatibilité et d'efficacité. Leur emploi devait être compatible avec une utilisation chez le petit animal.

Un résumé des différents protocoles testés est décrit ci-dessous.

Procédure de préparation des microsphères.

A l'aide d'une seringue montée d'une aiguille de petit diamètre, il fallait retirer du flacon de microsphères le plus de solution salée possible. Les microsphères en suspension étaient ensuite versées dans une seringue Hamilton avant l'injection cérébrale. Cette manipulation était assez dangereuse dans la mesure où les microsphères étaient conditionnées dans leur flacon sous vide. Il était donc difficile de retirer la solution salée compte-tenu de la pression sans se blesser avec l'aiguille. Il existait aussi des risques de contamination avec les agents cytotoxiques. Ce protocole de préparation n'était pas adapté au matériel dédié au petit animal.

Pour la deuxième formulation, il fallait également retirer du flacon de microsphères le plus de solution salée possible. La solution pure de microsphères était ensuite versée dans un tube BD Falcon™ de 10 ml. Pour les injections intracrâniennes, la solution était ensuite aspirée dans une seringue à tuberculine de 1cc tuberculin (Kendall Monoject) à l'aide d'une aiguille BD hypodermique 0,6x 25 de 23 G.

Procédure d'implantation stéréotaxique.

Nous avons testé différents types de micro-seringues et d'aiguilles compatibles avec l'implantation des microsphères mesurant de 100-300 µm.

- *Seringues Hamilton*

La première constatation était la différence importante en termes de concentration en microsphères en fonction du système utilisé. Les seringues Hamilton, habituellement employées pour les injections intracrâniennes chez le rat (10 µl-26G), n'aspiraient pas suffisamment la solution trop épaisse et le diamètre des aiguilles était trop petit (Photo 7). Ainsi, nous ne prélevions pas une dose suffisante de microsphères (Photo 8).

Photo 7: Seringue Hamilton classiquement utilisée pour les injections intracrâniennes chez le rat.

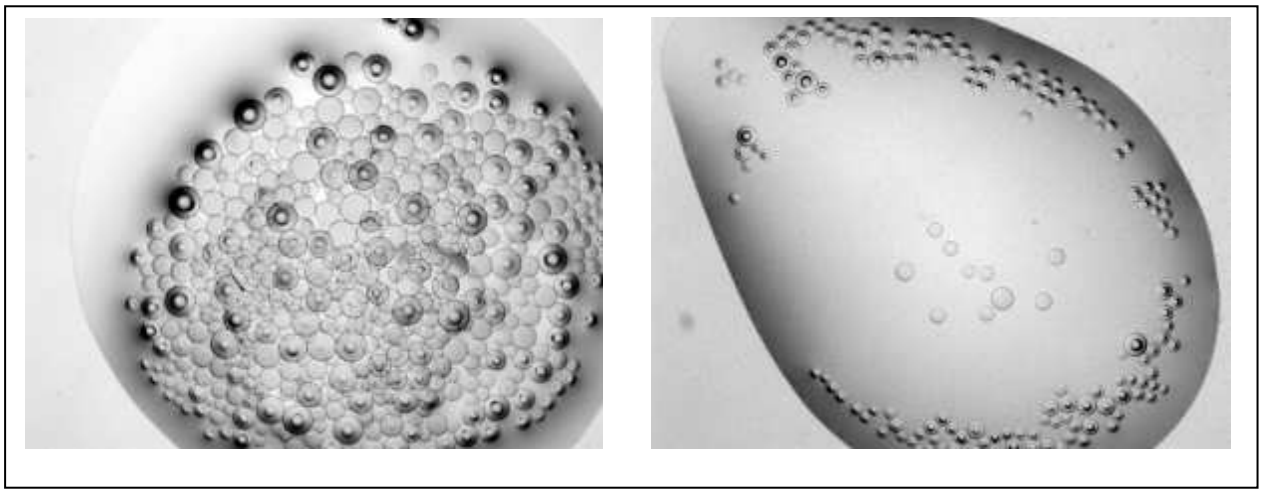


Photo 8: Différences de concentrations en microsphères selon la méthode de prélèvement utilisée. Gauche: Deux gouttes de 10µl de solution de microsphères provenant d'une pipette avec cône jaune; Droite: Deux gouttes de 10 µl de solution de microsphères provenant d'une seringue Hamilton (21G).

- *Convection-enhanced delivery*

Forts de notre expérience avec les nanocapsules lipidiques, nous avons essayé la technique de CED avec les microsphères afin d'éviter les reflux observés lors d'injection de composants de taille importante. Brièvement, le système consiste en une seringue Kendall Monoject 1 ml-23G connecté par un tube de polyéthylène (CoExTM EPPVC tubing, Harvard Apparatus, Les Ulis, France) à une deuxième seringue Kendall Monoject de 5 ml, montée sur une pompe osmotique (PHD 2000 Infusion, Harvard Apparatus, Les Ulis, France). Les connections étaient rendues étanches par l'application de 2-hydroxyéthylméthacrylate (Photo 9). Le débit d'injection était constant à 0.5µl/min. Les microsphères formaient des dépôts dans le système et obstruaient les tubes. La CED n'a pas été retenue pour l'injection des microsphères.

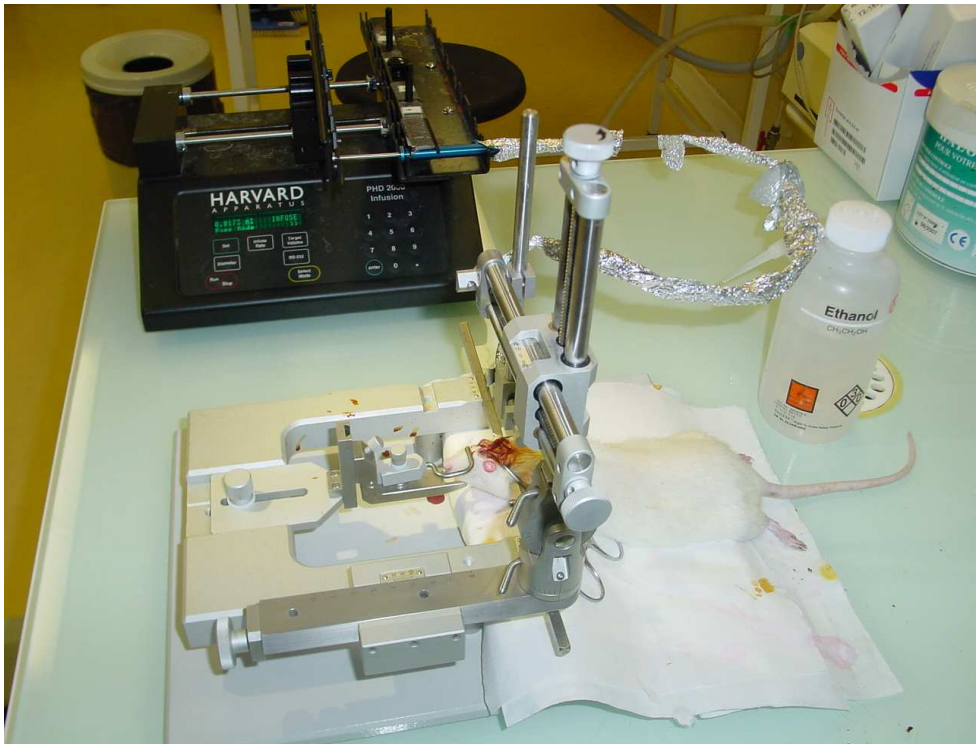


Photo 9: Montage de CED chez le rat pour les injections intracrâniennes.

- *Seringues à tuberculine (Kendall Monoject)*

Nous avons donc utilisé une seringue de diamètre plus important que celui des seringues Hamilton: une seringue à tuberculine de 1cc (Kendall Monoject) montée d'une aiguille BD Hypodermique 0.6x25mm de 23G (Photo 10).

Avec ce matériel, nous avons dû injecter 20 µl de solution et non 10 µl pour des raisons de graduation de la seringue. Le volume de 20 µl a déjà été injecté précédemment au laboratoire lors des études portant sur les microsphères de 5-FU (25), et nous savions que cette dose était bien tolérée. Nous avons donc gardé cette procédure d'injection pour nos microsphères de doxorubicine et d'irinotécan.

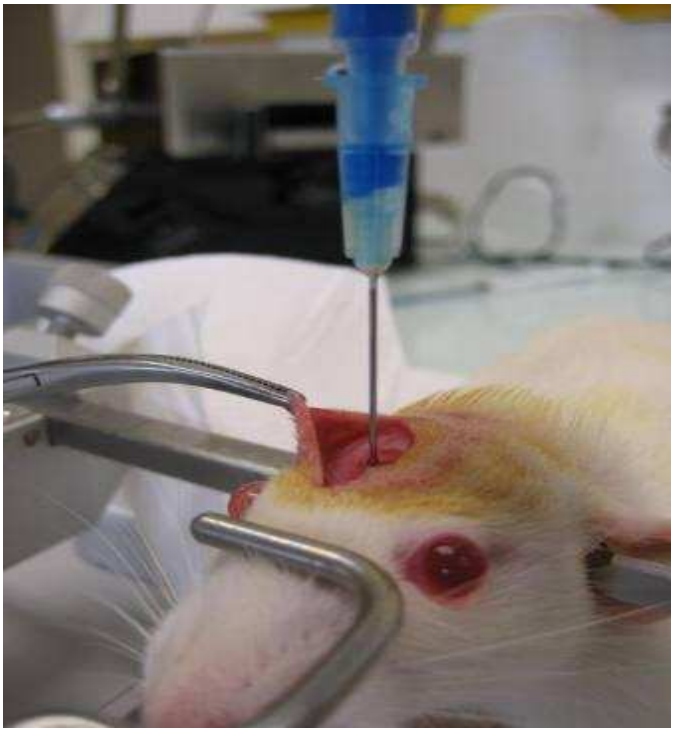


Photo 10: Injections intracrâniennes des microsphères blanches de Biocompatibles avec une seringue à tuberculine Kendall Monoject de 1 ml montée d'une aiguille BD Hypodermique 0,6x 25mm de 23G.

Nous avons commencé notre travail par une étude de biocompatibilité et ce pour les microsphères non chargées ou blanches, les microsphères de doxorubicine (appelées CM-BC1) et les microsphères d'irinotécan (appelées CM-BC2) puisque leur comportement au sein du parenchyme cérébral n'était pas connu.

Les cerveaux des rats de chaque groupe ont été analysés à plusieurs instants après l'injection de 20 µl de solution: à 8 jours, à 3 mois et à 6 mois. Ils ont tous été analysés à la recherche d'une réaction inflammatoire de rejet.

Une étude d'efficacité a été ensuite menée pour les CM-BC1 et les CM-BC2.

✓ **Article n°5**

- Local implantation of Doxorubicin Drug eluting Beads in rat glioma.
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Corresponding Author: Dr sandrine vinchon-petit, M.D.

Corresponding Author's Institution: Centre Paul Papin

First Author: sandrine vinchon-petit, M.D.

Order of Authors: sandrine vinchon-petit, M.D.; Delphine Jarnet; Sophie Michalak; Andrew Lewis; Jean-Pierre Benoît; Philippe Menei

Abstract: We evaluated the safety and the efficacy of Doxorubicin Drug Eluting Beads "CM-BC1" when used locally in a 9L glioma model. Twenty microlitres of 1 mg/ml CM-BC1 (4µg/rat), 10 mg/ml CM-BC1 (40µg/rat) or unloaded beads were injected into the brain of 27 rats which was analysed on day 8, month 3 or month 6. Then, after tumor implantation, rats were treated locally: (1) control group; (2) a group receiving 20 µl of unloaded beads, (3) a group "3 x 6 Gy whole-brain irradiation" (WBI), (4) a group receiving 20 µl of 1mg/ml CM-BC1 and (5) a group receiving 20 µl of 1mg/ml CM-BC1 followed by a WBI. Both the unloaded beads and the lower dose of 1 mg/ml CM-BC1 were well tolerated with no early deaths in opposite to 10 mg/ml CM-BC1. Medians of survival for the "1mg/ml CM-BC1" group and the combination group are respectively 28.9 and 64.4 days. These results were significant compared to the "unloaded beads" group. The rat's survival was not significantly improved in comparison with the radiotherapy group. This preliminary evidence suggests that 1mg/ml CM-BC1 could be interesting for recurrent high-grade gliomas. Further work is necessary to improve this seducing tool.

Suggested Reviewers:

Local implantation of Doxorubicin Drug eluting Beads in rat glioma.

S. Vinchon-Petit[1, 2, *], D. Jarnet[2], S. Michalak [3], A. Lewis [4], J-P Benoit[1] and P. Menei[1, 5.]

[1] INSERM, U646, Université d'Angers, Angers, F-49100, France.

[2] Centre Paul Papin, Angers, F-49100, France.

[3] Anatomopathology, CHU d'Angers, F-49933, Angers Cedex 9, France.

[4] Biocompatibles UK, Ltd., Farnham, Surrey, UK.

[5] Dpt of Neurosurgery, CHU d'Angers, F-49933, Angers Cedex 9, France.

*To whom correspondence should be addressed:

S. Vinchon-Petit, MD

Department of Radiation Therapy, Centre Paul Papin, 49933, Angers cedex 09, France.

Tel: (33) 241.35.29.16

Fax: (33) 241.35.27.35

E-mail: s.vinchon-petit@unimedia.fr

Conflict of Interest Statement

The manuscript submitted under multiple authorships was reviewed on the assumption that all listed authors concur with the submission and a copy of the final manuscript has been approved by the authors. Any conflict of interest was declared with this work.

ABSTRACT

We evaluated the safety and the efficacy of Doxorubicin Drug Eluting Beads "CM-BC1" when used locally in a 9L glioma model. Twenty microlitres of 1 mg/ml CM-BC1 (4µg/rat), 10 mg/ml CM-BC1 (40µg/rat) or unloaded beads were injected into the brain of 27 rats which was analysed on day 8, month 3 or month 6. Then, after tumor implantation, rats were treated locally: (1) control group; (2) a group receiving 20 µl of unloaded beads, (3) a group "3 x 6 Gy whole-brain irradiation" (WBI), (4) a group receiving 20 µl of 1mg/ml CM-BC1 and (5) a group receiving 20 µl of 1mg/ml CM-BC1 followed by a WBI. Both the unloaded beads and the lower dose of 1 mg/ml CM-BC1 were well tolerated with no early deaths in opposite to 10 mg/ml CM-BC1. Medians of survival for the "1mg/ml CM-BC1" group and the combination group are respectively 28.9 and 64.4 days. These results were significant compared to the "unloaded beads" group. The rat's survival was not significantly improved in comparison with the radiotherapy group. This preliminary evidence suggests that 1mg/ml CM-BC1 could be interesting for recurrent high-grade gliomas. Further work is necessary to improve this seducing tool.

Keywords: Glioma, Radiotherapy, Concomitant therapy, DC beads, Local chemotherapy, Doxorubicin.

1. INTRODUCTION

Malignant gliomas are the most common types of primary central nervous system tumors and have a growing incidence of 5-8/100 000 (Bauchet et al., 2007; Stupp R et al., 2005). Regardless of methods of treatment, most of these tumors recur locally. In an attempt to decrease these local recurrences, recent efforts have focused on designing polymer devices that deliver anti-tumor drugs into the resection surgical cavity. Macroscopic non biodegradable devices and more recently biodegradable wafers have been used for local chemotherapy of brain tumors in humans (Brem et al., 1995; Kubo et al., 1986; Oda et al., 1982; Valtonen et al., 1997; Westphal et al., 2003; Westphal et al., 2006). Efficacy of local chemotherapy with BCNU-wafers has been previously demonstrated in patients with recurrent glioblastoma and more recently in primary malignant glioma (Brem et al., 1995; Westphal et al., 2003; Westphal et al., 2006). But their size (several centimetres) doesn't allow a real intra-tumoral or intra-parenchymal implantation, neither a stereotactic administration. Moreover their incidence on survival is poor. Many other drug delivery devices have been developed and evaluated in animal models (Krauze et al., 2007; Kubo et al., 1986; Lesniak et al., 2005; Rousseau et al., 2009; Vauthier et al., 2003; Mu et al., 2003; Bartoli et al., 1990; Menei et al., 1996). Nevertheless, very few have come to clinical trial (Menei et al., 2004; Menei et al., 2005a; Sapin et al., 2006). One potential strategy is to test microparticles in suspension. Due to their size (1 to 1000 µm), they can be implanted easily in discrete, precise and functional areas of the brain, using needles as narrow as 21 gauge, without causing damage to the surrounding tissue (Menei et al., 2005b).

One such device that is currently the subject of widespread clinical investigations is a drug delivery embolisation system produced from a biocompatible polyvinyl alcohol (PVA) hydrogel known as DC Bead®, or LC Bead® in the USA (Biocompatibles, UK Ltd). This device is indicated for the treatment of hypervascular tumors (Aliberti et al., 2006; Aliberti et al., 2008; Eyol et al., 2008; Malagari et al., 2008; Fiorentini et al., 2007; Gonzalez et al., 2008; Lewis et al., 2006a; Lewis et al., 2006b; Lewis et al., 2007; de Baere et al., 2008; Lencioni et al., 2008;

Poon et al., 2007; Tang et al., 2008; Forster et al., 2010; Keese et al., 2009) and is being used to treat both primary and metastatic liver cancer. Outside of its use as a drug delivery embolisation system, DC Bead has also been shown in preclinical models to have the potential for treatment of other tumors such as pancreatic cancer (Forster et al., 2010) and peritoneal carcinomatosis (Keese et al., 2009), by local direct injection of the microspheres. DC Bead microspheres studied in this paper are produced from a biocompatible polyvinyl alcohol (PVA) hydrogel that has been modified with sulphonate groups for the controlled loading and delivery of chemotherapeutic drugs. DC Bead has been developed in order to deliver a local and sustained dose of drug direct to the tumor over a protracted period of time, whilst occluding the feeding vessels of the tumor. Because doxorubicin is known to be efficient against brain tumors, using doxorubicin-loaded DC beads could be a new strategy to reduce glioma volume (Stan et al., 1999). The purpose of this project was to study the long term biocompatibility and toxicity of doxorubicin-loaded DC beads in the rat brain. We also evaluated the efficiency of doxorubicin-loaded beads on a rat glioma model, alone or associated to a fractionated radiation therapy.

2. MATERIALS AND METHODS

2. 1. Drug-eluting bead resuspension preparation

Unloaded Beads (DC Bead) and Doxorubicin loaded Drug Eluting Beads (CM-BC1) were provided by Biocompatibles UK Ltd, Surrey, UK. A bead size range of 100-300 µm beads were used for these studies. All manipulations were made in a special room dedicated to cytotoxics handling under a fume extraction hood.

Two loading levels in the CM-BC1 were studied: 1 mg/ml and 10 mg/ml CM-BC1 provided lyophilized and sterile.

Beads were rehydrated in the vial. Five minutes after the addition of 1 ml of sterile water, the mixture was decanted in a BD Falcon™ 10 ml Conical Tube. The vial was rinsed with 1 ml of sterile water and this was added to the other 1 ml in the BD Falcon™ 10 ml Conical Tube. Finally, the bead suspension was mixed with 3ml 0.6% alginate solution, to reach a final volume of 5 ml. The alginate used for suspension and viscosity increase was ultra-pure Phycomer E01 (CellMed AG, Alzenau, Germany) which is mannuronic acid rich and has a molecular weight of approximately 800,000. For the intracranial injection, we had the solution was drawn into a Kendall Monoject syringe with a BD Hypodermic 0,6x 25mm 23G- needle.

2. 2. DC Beads brain biocompatibility and toxicity

DC Beads were analyzed for their biocompatibility in brain parenchyma before therapeutic studies. After intraperitoneal anesthesia by xylazine (Rompun, Bayer, Puteaux, France) (10mg/kg) and ketamine (Clorkétam, Vétoquinol, Lure, France) (50mg/kg), rats were placed in a small-animal stereotactic frame (Kopf Instruments, Phymep, France). After shaving and skin disinfection, sagittal incision of 2 cm was made to expose the skull, followed by a burr hole 0.5 mm anterior and 3 mm lateral from the bregma using a small drill. Twenty microlitres of beads resuspension were injected manually with a rate of 0,5 µl/min, 5 mm deep in right striatum of 27 female Wistar rats (according to the Paxinos atlas) with a Kendall Monoject syringe and a

BD Hypodermic 0.6x 25 23G-needle . After a final wait of 5 min, the needle was removed and the wound was sutured.

Groups were as follows: (1) a unloaded beads group (control group) (n = 9); (2) a 1 mg/ml CM-BC1 group (n = 9); (3) a 10 mg/ml CM-BC1 group (n = 9). Rats were examined daily. Three animals were scheduled to be sacrificed (atmosphere saturated with CO_2) at each time point, on day 8, month 3, and month 6. The brain was surgically removed, fixed, dehydrated and paraffin embedded. Standard staining (hematoxylin and eosin (H&E)), Perls coloration (for siderophages staining), Lugol Blue staining (for myelin) and Kossa staining (for microcalcifications) were performed. The immunohistochemistry (IHC) stains were used are shown in table 1. The histology was reported in a descriptive manner, in particular to evaluate the successful implantation of the beads, the location / distribution of the beads in the brain and the brain tissue reaction.

2. 3. Tumor cell line

Rat 9L-glioma cells (European Collection of Concealment Culture, n° 94110705, Salisbury, U.K.) were cultivated in the “DMEM” medium (“Dulbecco's Modified Eagle's Medium”, Biowhittaker, Verviers, Belgium) added with 10% of foetal calf serum (FBS, Biowhittaker) and of a mixture of antibiotics: penicillin (100 UI/ml), streptomycin (0.1 mg/ml) and amphothericin B (25 µg/ml) (ABS, Sigma, Saint Quentin Fallavier, France). Cells were maintained in a balanced wet atmosphere (37 °C and CO_2 5%). Cells were ready to be used to induce a brain tumor, after trypsinisation (trypsin/EDTA (Sigma)) and resuspension in “EMEM” (“Eagle’s Minimum Essential Medium”, Biowhittaker).

2. 4. Animals and intracranial tumor implantation

Female Fischer-F344 rats were obtained from Charles River Laboratories France (L’Arbresle, France). Ten weeks-old, they weighted 150 to 200g. They were housed in groups of 4 in cages in conformity with the standards of the directives of the Union European

and dealt with by the animal facilities of the Faculty of Medicine of Angers, establishment approved according to the law.

Ten microlitres of 10^3 9L-cells suspension were implanted by stereotactic conditions with a 10 µl -26G Hamilton syringe (Harvard Apparatus, Ulis, France) into the right striatal region of the rat brain as describe above for beads implantation.

2. 5. Therapeutic protocol

On day 6 after tumor cells implantation, rats were assigned into 5 experimental groups and 20 µl of beads suspension were injected according the same coordinates as the 9L cells. Groups were as follows: (1) control group (n = 8); (2) a group receiving one injection of unloaded beads (n = 7), (3) a group only irradiated by a whole-brain irradiation (WBI) with a total dose of 18Gy, (4) a chemotherapy group, receiving one injection of 1mg/ml CM-BC1 and (5) a chemotherapy plus radiotherapy group, receiving one injection of 1mg/ml CM-BC1 followed by WBI for a total dose of 18Gy. Groups 4 and 5 received 4 µg of doxorubicin. Radiotherapy was administered on days 8, 11 and 14 to animals in groups 3 and 5.

2. 6. Animal observation

Rats were examined daily and staged for activity and well-being according to a classification developed in our animal facilities (data not published). Rats too weak to feed and to stand (corresponding to stage 2) were sacrificed. The day of euthanasia was recorded and used in the survival analysis. Rats were weighed weekly.

2. 7. Statistics

Survival was calculated from the day of the tumor implantation and presented as median and mean ± SE (Standard Error). The student t-test comparing combination treatment groups was performed. SPSS® software was used for that purpose and tests were considered as significant with p values <0.05. Any rat surviving longer than 120 days was regarded as a ‘long survivor’. The Kaplan-Meier method was used to plot animal survival.

3. RESULTS

3.1. Biocompatibility and safety of Bead implantation in the brain

The rat brains were evaluated for brain tissue reactions as a result of the implantation. For the “unloaded control beads” group, there was no unexpected mortality. Clinical staging and weight curves were normal for all animals until sacrifice. There was no issue with wound healing following the procedure. For the 9 rats analyzed, the pack of beads was localized in the striatum. It is important to note that in all the slides, some beads were displaced from their implantation site during the slicing, with sometimes a tearing of the surrounding tissue, probably due to the difference in consistency between brain tissues and beads. Their location was still perfectly visible as a round and well limited hole in the tissue. No beads were in the subarachnoidian space. The beads appeared round, and stained similar to basophile material (H&E staining), striated by the effect of the microtome knife. This is a sign of a hard material (these soft, hydrated beads become dehydrated during the tissue fixing process). There are no signs of deformation, degradation or vacuolization of the beads over time (Fig. 1A). The striking point is the total lack of a foreign body reaction around the beads on H&E staining (Fig. 1B). Immunohistochemistry showed a very weak, non-specific, astrocytic reaction due to the implantation. This lack of strong astrocytic reaction supports the supposition that the biocompatibility of the material in the brain is excellent. There was no lymphocytic infiltration, nor demyelinisation. Neuronal markers showed healthy neurons and neuronal fibers in close proximity to the beads.

For the “1 mg/ml CM-BC1” group, rats received 4 µg of doxorubicin per dose of beads. All rats presented a good neurological and general state before sacrifice. Animals were sacrificed at day 8, month 3 and month 6. There was no issue with wound healing in any of these rats. There was no evidence of necrosis or haemorrhage on any of the macroscopic examinations. It is important to note that for all the sections, as for the control beads, some beads were displaced from their implantation site during the slicing, again, with some tearing of the surrounding tissue

on occasion, due to brain tissue and bead differences. Their localization was still perfectly visible as a round and well limited hole in the tissue. The beads (or their localization) were exactly in the predefined target in the middle of the striatum for most animals. They were localized slightly more medially in the striatum for one animal, opening the lateral ventricle, without hydrocephaly. In one animal, one bead was observed on the cortical point of injection. Except for this one, there were no beads in the subarachnoidian space in any of the observed slides. The beads appeared exactly as the unloaded control beads: round, stained similarly to basophile material (H&E staining) and striated by the effect of the microtome knife. On H&E staining, there was a moderate cell reaction with a moderate inflammatory reaction in two animals (one at month 3, one at month 6). There was no cell loss and no anomaly of brain architecture in any of the observed animals (Fig.2).

For the “10 mg/ml CM-BC1” group, rats received 40 µg of doxorubicin per dose of beads. On day 5, neurological condition of two rats started to deteriorate. Those two rats died spontaneously: one on day 9 and one on day 21 (animals were found dead in the morning and histological examination was not possible). Seven rats were too weak to feed and to stand (stage 2) and were sacrificed 21 days after the bead implantation according to the protocol. The 10th rat was still alive at 3 months and was sacrificed according to the protocol. There was no issue with wound healing. Moderate oedema was evident in 7 rats, in addition to significant necrosis with haemorrhage at the level of the injection site and into the adjacent cortex. For the 8 rats analyzed, beads were observed in the middle of the striatum in 6 animals. No beads were visible in two animals, one because slicing problems and another probably due to unsuccessful bead implantation. As for the group 1, some beads were displaced from the implantation site during the slicing, with sometimes a tearing of the surrounding tissue, probably due to the difference in consistency between brain tissues and beads. Their localization was still perfectly visible as a round and well limited hole in the tissue. For each rat assessed, microscopic examination showed significant oedema, necrosis (sometimes calcified, with calcifications confirmed by a Kossa

staining), and haemorrhage around the 10 mg/ml CM-BC1. This necrosis was surrounded by a marked cell reaction (macrophages, with some T lymphocytes), confirmed by Perls reaction, and neoangiogenesis, indicated by proliferation of abnormal microvessels with an enlarged wall. The brain tissue around this cell reaction was vacuolated (Fig.3). For all the animals, GFAP IHC staining showed a lack of astrocytes in contact with the beads and an astrocytic reaction in the surrounding area. Neurofilament, NeuN and synaptophysin immunostaining showed the disappearance of neurons and axons in a large area around the implantation site, indicating massive neuronal death. For rat 10 which was still alive and sacrificed at month 3, macroscopic and microscopic examinations showed the lack of beads, probably due to a technical problem during the implantation procedure.

3. 2. Combined effects of chemotherapy and radiotherapy

After the biocompatibility study, rats were randomized in only five experimental groups. We decided not to use 10 mg/ml CM-BC1 because of their brain toxicity. All non-treated rats died before d35, with a median and mean survival of 27 and 28.1 days (Fig. 4 and Table 2). Similar survival was observed in animals that received unloaded control beads, with all animals dying by day 31 (p = 0.16 versus control). Animals receiving WBI had an extended survival compared to groups 1 and 2, with a median survival of 49.5 days. There were 2 long term survivors in this group.

Animals in group 4 (1 mg/ml CM-BC1) all died by day 33, with a median survival of 28 days. The survival was not significantly different from untreated animals. When 1 mg/ml CM-BC1 was combined with WBI (group 5), there was a significant increase in median survival (53 days) compared to unloaded beads or CM-BC1 alone. In this group there were two long term survivors. The association gives the best median of survival compared to the “WBI” group with 64.4 days versus 59.9 days. Unfortunately, this difference is not significant. We noted that in the combined group, there were early deaths. Some rats died before the untreated animal and 30% of

the animals died before the first rats died in “WBI” group. Survival curves for all groups were obtained by Kaplan-Meier Method, including long survivor animals (Fig. 4).

4. DISCUSSION

DC Bead microspheres have been particularly studied as anticancer agents against hepatic tumors (Aliberti et al., 2008; de Baere et al., 2008; Eyol et al., 2008; Gonzalez et al., 2008; Lencioni et al., 2008; Lewis et al., 2006a; Lewis et al., 2006b; Malagari et al., 2008; Poon et al., 2007). The incorporation of doxorubicin in this device was performed to enhance the cytotoxicity of this type of molecule when administered locally. Its direct administration could improve the efficiency of the chemotherapeutic agent, as the slow release of the drug allows a prolonged contact between tumor and chemotherapeutic agent. In preliminary studies, levels of doxorubicin were still retained even at 90 days (Lewis et al., 2006a; Lewis et al., 2007). DC Beads present advantages compared to the others techniques designed for direct instillation into the tumor bed such as wafer implants, conjugated nanoparticles, liposome encapsulated drug, or intratumoral infusion with Ommaya reservoir. Firstly, there have been already more than 10,000 procedures carried out with this doxorubicin bead combination in the treatment of liver tumors, demonstrating their safety, and also a Phase II randomised control trial which shows lower side effects and benefits for advanced patients (Lammer et al., 2010). Second, there have been a number of pharmacokinetic studies which demonstrate the release of dox into the bloodstream is very low compared to the traditional treatments and also preclinical work which demonstrates the release of drug occurs over many weeks (Namur et al., 2010). Doxorubicin beads have been well characterised *in vitro* with respect to drug loading and elution kinetics and effects on the physical attributes of the beads related to catheter delivery. This data could have been interesting for a sustained release during all the radiation therapy time for a concomitant effect. If other drug delivery devices have been developed and evaluated in animal models, very few are supported by so encouraging data. In our study, 10 mg/ml CM-BC1 (total dose 40 µg of doxorubicin per rat) were highly clinically toxic. The lower dose of 1 mg/ml CM-BC1 (total dose of 4 µg of

doxorubicin per rat) was well tolerated with no early deaths. In one publication describing local therapy in a rat glioma model, doxorubicin was loaded in polyanhydride polymers (PCPP-SA). PCPP-SA were prepared using the mix-melt method (Lesniak et al., 2005. 2005). It was found that the doses of 1mg, 700µg and 500µg of doxorubicin were toxic, whereas 300µg was well tolerated. The type of toxicity observed was the same as in our study, namely necrosis and oedema. It is important to note that this polyanhydride polymer allows a slow release of the drug: 1% loaded polymer released 6.5% of drug over 200 hours, and 10% loaded polymer released 21% of drug over 200 hours. This release, slower than that obtained with the DC-beads, could explain the differences in term of tolerance over different dose ranges. While the in vitro potency of doxorubicin is remarkable and its current indications in treating peripheral tumors have proven efficacious, doxorubicin is highly toxic and presents a very narrow therapeutic window.

The other purpose of this study was to assess the anti-tumor activity, in terms of survival benefit, of administration of drug-eluting beads in rats bearing a cerebrally implanted syngeneic glioblastoma 9L. The potential added effect of radiotherapy was also evaluated. In terms of survival, the control groups (untreated and unloaded beads) median survival was as expected for this animal model in the laboratory (27 and 25 days, respectively), with a small standard error of the mean confirming the quality and reproducibility of the model. Although the 9L gliosarcoma is not a perfect model of human glioma because it is less invasive, we used it because of its ability to create very similar sized tumors. CM-BC1 (4 µg/rat) in combination with radiotherapy significantly increased the median survival, compared to the chemotherapy alone group and there were two long survivors for this group. However it was not significantly different, in comparison with the radiotherapy alone group. The major finding of this work is that doxorubicin beads, administered in combination with external beam irradiation, resulted in a significant enhancement in median survival time compared to the chemotherapy group (p=0.02). The association gives the best median of survival, even compared to the “WBI” group with 64.4 days versus 59.9 days even though this result is not significant.

This work gives interesting preliminary results because the behaviour of beads in rat brain was unknown until now. In this study, doxorubicin-loaded beads were mixed with a solution of ultra-pure high molecular weight alginate. Alginate, a viscous polymer derived from brown algae, is an established compound in many biomedical and nutritional applications. Due to alginate's biocompatibility and simple gelation with divalent cations such as Ca^{2+}, it is widely used for cell immobilization and encapsulation. Mixing the alginate with the beads prior to intracerebral implantation was performed in order to minimize displacement of the beads. As we observed in biocompatibility studies, we have little back flow with this technique since only a few beads can be observed in the subarachnoid space in a few animals. A pilot study by Brinker *et al* reported on the investigation of DC Bead with doxorubicin or irinotecan in a B4TCa rat glioma model (Blates et al., 2010). While both drugs were efficacious in treating the tumor, doxorubicin was shown to be far more toxic than irinotecan and the method of microsphere implantation was also not readily translatable into the clinic.

Our work demonstrates that DC Beads are well tolerated in rat brain and can be easily used in patients when combined with the alginate carrier.Further work is now necessary to improve this concept for better preclinical results. We have the opportunity to change the concentration of the loaded therapeutic agent or to increase their volume of distribution for example. Others drugs could also be an option because DC Beads can be loaded with other interesting therapeutic agents, such as irinotecan, topotecan and mitoxantrone (Lewis et al., 2009). They have shown the same safety and efficiency in patients for hepatic tumors and irinotecan can be efficient against glioma cells (Buie et al., 2008).

5. CONCLUSION

This preclinical evidence suggests that 1mg/ml CM-BC1 may be easily used to treat high-grade malignant brain tumors. Our data represent the first demonstration of the compatibility between these compounds and rat brain, and potentially indicate a therapeutic

option for this class of tumors which often present problems of accessibility and therapeutic resistance.

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LEGENDS

Table 1: Immunohistochemistry stains

Table 2: Descriptive and statistical data from the survival study depending on groups of treatment.

Fig. 1A: Unloaded beads in rat right striatum on day 8 (H&E staining x 40).

Fig. 1B: Unloaded beads in rat right striatum on month 6 (NeuN staining x 2.5).

Fig. 2: 1 mg/ml CM-BC1 in rat right striatum on month 6 (H&E staining x 20).

Fig. 3: 10 mg/ml CM-BC1 in rat right striatum on day 21 (HES x 20).

Fig. 4: Survival Curves were obtained by Kaplan-Meier Method.

Table(s)

1-Vinchon

Antibodies anti-	Company	Clone	Dilution	Staining
CD3	Dako	Poly	100	T lymphocytes
GFAP[a]	Dako	Mono 6F2	900	Astrocytes
Neu N	Zymed	Mono A60	100	Neuronal bodies
Neurofilament	Monosan	Mono2F11	120	Axons
Synaptophysine	Biogenex	Mono Snp88	200	Neurons and their axons

a) GFAP: Glial fibrillary acidic protein

Table 1: Immunohistochemistry stains

GROUPS (n)	Range (days)	Median of survival (days)	Mean time of survival (days) ±SE	Mean ILT (%)	Long term survivors
Group « control » (*n*=8)	24-35	27.0	28.1 ±1.33	-	0
Group «unloaded beads » (*n*=7)	24-31	25.0	25.7±0.92	-	0
Group «WBI» (*n*=10)	33-120	49.5	59.9±2.85	133	2
Group «1mg/ml CM-BC1 » (*n*=10)	26-33	28.0	28.9±0.95	12.4	0
Group «1mg/ml CM-BC1+WBI » (*n*=10)	21-120	53.0	64.4±11.62	133.1	2

Table 2: Descriptive and statistical data from the survival study depending on groups of treatment. Group 1: group "control"; group 2: "unloaded beads"; group 3: "WBI (whole brain irradiation)"; group 4: "1mg/ml CM-BC1" and group 5: "1mg/ml CM-BC1 + WBI" of 18 Gy (3x6 Gy).

Increase in survival time (ILT) is calculated in comparison to the "unloaded beads" group (%). *SE* means "Standard Error".

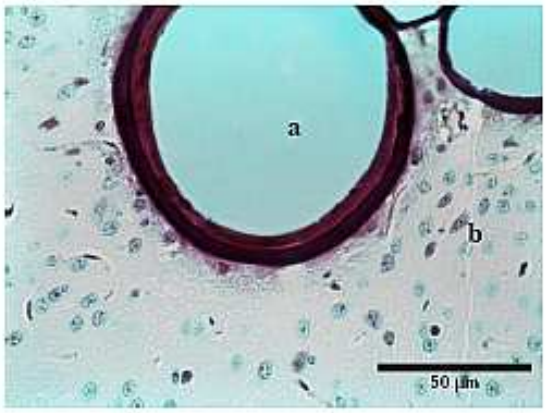

Fig. 1A: Unloaded beads in rat right striatum on day 8 (H&E staining x 40).

a: Unloaded beads. b: Respected neuronal nucleus.

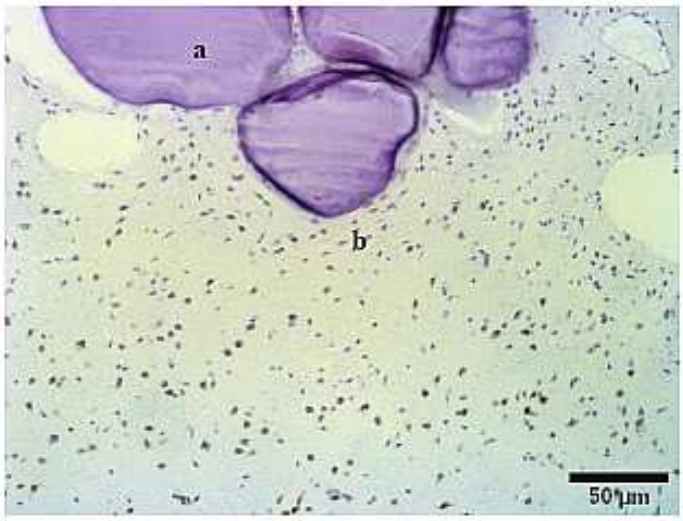

Fig. 1B: Unloaded beads in rat right striatum on month 6 (NeuN staining x 2.5).

a: Unloaded beads. b: Respected neuronal nucleus.

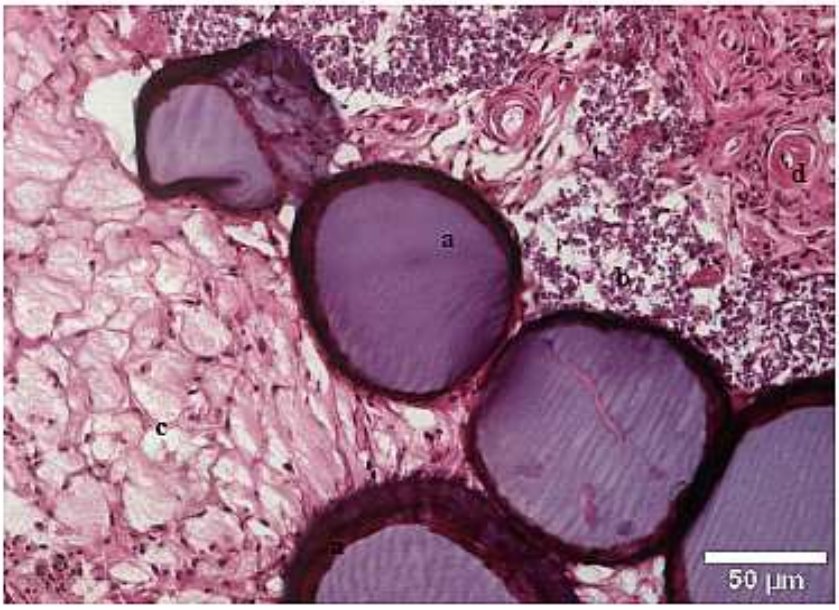

Fig. 3: 10 mg/ml CM-BC1 in rat right striatum on day 21 (HES x 20).

a: 10 mg/ml CM-BC1; b: Necrosis; c: Vacuolisation of brain tissue;

d: Neoangiogenesis (with abnormal vessels).

Figure(s)

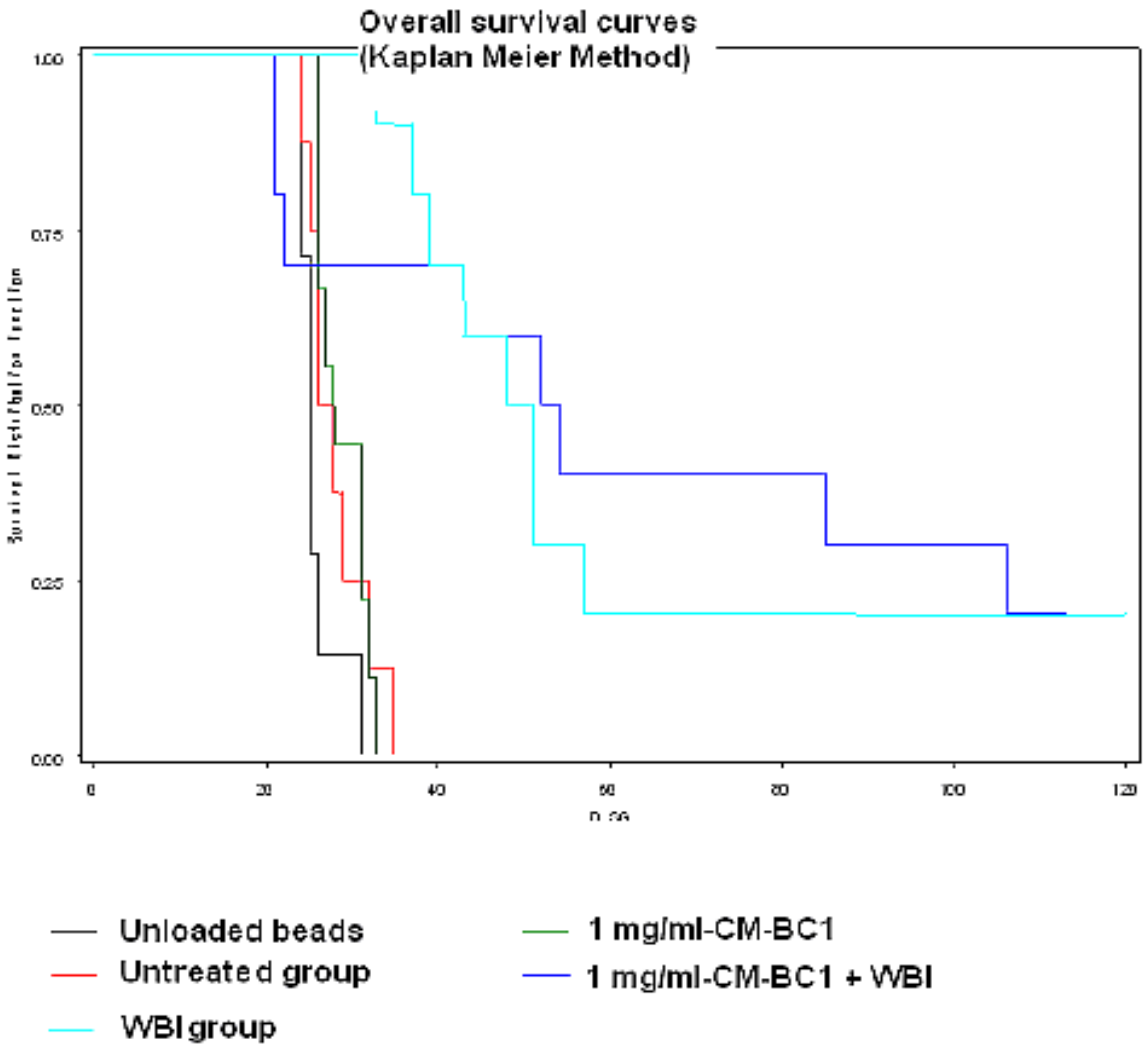

Fig. 4: Survival Curves were obtained by Kaplan-Meier Method.

✓ **Article n°6**

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Local implantation

of Irinotecan Drug Eluting Beads for 9L-rat glioma

S. Vinchon-Petit[1, 2], D. Jarnet[2], S. Michalak[3], A. Lewis[4], J-P Benoit[1] and P. Menei[1, 5, *]

[1] INSERM, U646, Université d'Angers, F-49100, Angers, France.

[2] Centre Paul Papin, F-49933, Angers Cedex 9, France.

[3] Anatomopathology, CHU d'Angers, F-49933, Angers Cedex 9, France.

[4] Biocompatibles UK, Ltd., Farnham, Surrey, UK.

[5] Dpt of Neurosurgery, CHU d'Angers, F-49933, Angers Cedex 9, France.

*To whom correspondence should be addressed:

Sandrine Vinchon-Petit, MD.

Department of Radiation Therapy, Centre Paul Papin 49933, Angers cedex 09, France.

Tel : (33) 241.35.29.16

Fax: (33) 241.35.27.35

E-mail: s.vinchon-petit@unimedia.fr

INTRODUCTION

Malignant gliomas are the most common types of primary central nervous system tumors and have a growing incidence of 5-8/100 000 (1; 2). Regardless of methods of treatment, most of these tumors recur locally. In an attempt to decrease these local recurrences, recent efforts have focused on designing polymer devices that deliver anti-tumor drugs into the surgical resection cavity. Efficacy of local chemotherapy with BCNU-wafers has been previously demonstrated in patients with recurrent glioblastoma and in primary malignant glioma (3-8). The limitation of these implants is their size (several centimetres), which does not allow a real intra-tumoral or intra-parenchymal implantation, neither a stereotactic administration. Microparticles in suspension, due to their size (1 to 1000 µm), can be easily implanted by these techniques in functional areas of the brain, without causing damage to the surrounding tissue (9). Many microparticular drug delivery devices have been developed and evaluated in animal models (10-17). Nevertheless, very few have come to clinical trial (18-20). One such device that is currently the subject of widespread clinical investigations is a drug delivery embolisation system produced from a biocompatible polyvinyl alcohol (PVA) hydrogel known as DC Bead™, or LC Bead™ in the USA (Biocompatibles, UK Ltd). This device is indicated for the treatment of hypervascular tumors (21-30) and is being used to treat both primary and metastatic liver cancer. Outside of its use as a drug delivery embolisation system, DC Bead™ has also been shown in preclinical models to have the potential for treatment of other tumors such as pancreatic cancer (31) and peritoneal carcinomatosis (32), by local direct injection of the microspheres. A pilot study by Brinker *et al* reported on the investigation of DC Bead with doxorubicin or Irinotecan in a B4TCa rat glioma model (33). While both drugs were efficacious in treating the tumor, doxorubicin was shown to be far more toxic than irinotecan and the method of microsphere

implantation was also not readily translatable into the clinic. We have most recently reported on a complimentary study to this B4TCa model, in which we evaluated the safety and the efficacy of an encapsulated chemotherapy formulation "CM-BC-1" locally implanted in a 9L glioma model in combination with whole-brain radiation (34). In this study, CM-BC-1 consists of PVA hydrogel microspheres (DC Bead) preloaded with doxorubicin and suspended in an alginate viscosity-modifying solution to allow it to be delivered directly intratumorally. Two doxorubicin loading levels in the CM-BC1 were studied: 1 mg/ml and 10 mg/ml CM-BC1. The lower dose of 1 mg/ml CM-BC1 was well tolerated but 10 mg/ml CM-BC1 showed some toxic effects. Combination of 1mg/ml-Doxorubicin beads and whole brain irradiation (WBI) improved survival in comparison with the chemotherapy procedure (p=0.02). Unfortunately, the difference was not significant between the WBI group and the combination group.

This new work aimed to find a drug that was "less toxic" than the doxorubicin effect and more efficient. Irinotecan Eluting Beads are also under investigation for the treatment of patients with metastatic colorectal cancer (27-30). Pursuing the work began with CM-BC1 and by considering the impressive rates of response with irinotecan alone or in combination with bevacizumab in several phase I studies, the use of the locally-implanted CM-BC2 (PVA hydrogel beads preloaded with irinotecan and suspended in an alginate delivery solution) could be a new strategy to reduce glioma volume (35-37). We studied the long term biocompatibility and toxicity of CM-BC2 in the rat brain and we evaluated the efficiency of CM-BC2 on a rat glioma model, alone or with WBI.

MATERIALS AND METHODS

1. Drug-eluting bead resuspension preparation

Unloaded Beads (DC Bead) and preloaded Irinotecan Drug Eluting Beads were provided by Biocompatibles UK Ltd, Surrey, UK. A bead size range of 100-300 µm were used for these studies. All manipulations were made in a special room dedicated to cytotoxics handling under a fume extraction hood.

One irinotecan loading level in the preloaded beads was provided for this study: 100 mg/ml, provided lyophilized and sterile in a vial ready for mixing with the alginate solution just prior to use to form the final bead suspension formulation, CM-BC-2.

Beads were rehydrated in the vial. Five minutes after the addition of 1 ml of sterile water, the mixture was decanted in a BD Falcon™ 10 ml Conical Tube. The vial was rinsed with 1 ml of sterile water and this was added to the other 1 ml in the BD Falcon™ 10 ml conical tube. Finally, the bead suspension was mixed with 3ml 0.6% alginate solution, to reach a final volume of 5 ml- the CM-BC-2 formulation. The alginate used for suspension and viscosity increase was ultra-pure Phycomer E01 (CellMed AG, Alzenau, Germany) which is mannuronic acid rich and has a molecular weight of approximately 800,000. For the intracranial injection, the solution was drawn into a 1cc syringe (Kendall Monoject) with a BD Hypodermic 0.6x 25mm 23G- needle.

2. CM-BC2 brain biocompatibility and toxicity

CM-BC2 was analyzed for its biocompatibility in brain parenchyma before therapeutic studies. Twenty microlitres of bead resuspension were injected into the right striatum of 18 female Wistar rats. After intraperitoneal anesthesia by xylazine (Rompun,

Bayer, Puteaux, France) (10 mg/kg) and ketamine (Clorkétam, Vétoquinol, Lure, France) (50mg/kg), rats were placed in a small-animal stereotactic frame (Kopf Instruments, Phymep, France). After shaving and skin disinfection, sagittal incision of 2 cm was made to expose the skull, followed by a burr hole 0.5 mm anterior and 3 mm lateral from the bregma using a small drill. Suspensions were injected 5 mm deep in the striatum (according to the Paxinos atlas) with a 1cc syringe (Kendall Monoject) and a BD Hypodermic 0.6x 25 23G-needle. After a final wait of 5 min, the needle was removed and the wound was sutured.

Groups were as follows: (1) an unloaded beads group (control group) (n = 9) and (2) a 100 mg/ml CM-BC2 group (n = 9). Rats were examined daily. Three animals were scheduled to be sacrificed (atmosphere saturated with CO_2) at each time point, on day 8, month 3 and month 6. The brain was surgically removed, fixed, dehydrated and paraffin embedded. Standard staining (hematoxylin and eosin (H&E)), Perls coloration (for siderophages staining), Lugol Blue staining (for myelin) and Kossa staining (for microcalcifications) were performed. The immunohistochemistry (IHC) stains were used are shown in table 1. The histology was reported in a descriptive manner, in particular to evaluate the successful implantation of the beads, the location / distribution of the beads in the brain and the brain tissue reaction.

3. Tumor cell line

Rat 9L-glioma cells (European Collection of Concealment Culture, n° 94110705, Salisbury, U.K.) were cultivated in the "DMEM" medium ("Dulbecco's Modified Eagle's Medium", Biowhittaker, Verviers, Belgium) added with 10% of foetal calf serum (FBS, Biowhittaker) and of a mixture of antibiotics: penicillin (100 UI/ml), streptomycin (0.1 mg/ml) and amphothericin B (25 µg/ml) (ABS, Sigma, Saint Quentin Fallavier, France). Cells were maintained in a balanced wet atmosphere (37 °C and CO_2 5%). Cells were ready to be

used to induce a brain tumor, after trypsinisation (trypsin/EDTA (Sigma)) and resuspension in "EMEM" ("Eagle's Minimum Essential Medium", Biowhittaker).

4. Animals and intracranial tumor implantation

Female Fischer-F344 rats were obtained from Charles River Laboratories France (L'Arbresle, France). Ten weeks-old, they weighed 150 to 200g. They were housed in groups of 4 in cages in conformity with the standards of the directives of the Union European and dealt with by the animal facilities of the Faculty of Medicine of Angers, establishment approved according to the law.

Ten microliters of 10^3 9L-cells suspension were implanted by stereotactic conditions with a 10 µl -26G Hamilton syringe (Harvard Apparatus, Ulis, France) into the right striatal region of the rat brain as describe above for beads implantation.

5. Therapeutic protocol

On day 6 after tumor cells implantation, rats were assigned into 5 experimental groups and 20 µl of beads suspension were injected according the same coordinates as the 9L cells. Groups were as follows: (1) control group ($n = 8$); (2) a group receiving one injection of unloaded beads ($n = 7$), (3) a group only treated with a whole-brain irradiation (WBI) to a total dose of 18 Gy, (4) a chemotherapy group, receiving one injection of 100 mg/ml CM-BC2 and (5) a chemotherapy plus radiotherapy group, receiving one injection of 100 mg/ml CM-BC2 followed by WBI (18 Gy). Groups 4 and 5 received a total dose of 400 µg of irinotecan. Radiotherapy was administered on days 8, 11 and 14 to animals in groups 3 and 5.

6. Animal observation

Rats were examined daily and staged for activity and well-being according to a classification developed in our animal facilities (data not published). Rats too weak to feed

and to stand (corresponding to stage 2) were sacrificed. The day of euthanasia was recorded and used in the survival analysis. Rats were weighed weekly.

7. Statistics

Survival was calculated from the day of the tumor implantation and presented as median and mean ± SE (Standard Error). The Kaplan-Meier method was used to plot animal survival and the Log rank test to compare survivals. Student t-test was performed to compare means of survival. SPSS® software was used for that purpose and tests were considered as significant with p values <0.05. Any rat surviving longer than 120 days was regarded as a 'long survivor'.

RESULTS

1. Biocompatibility and safety of brain implantation of CM-BC-2

The rat brains were evaluated for brain tissue reactions as a result of the implantation. For the "unloaded beads" group, there was no unexpected mortality. Clinical staging and weight curves were normal for all animals until sacrifice. There was no issue with wound healing following the procedure. For the 9 rats analyzed, the pack of beads was localized in the striatum. It is important to note that in all the slides, some beads were displaced from their implantation site during the sectioning, with sometimes a tearing of the surrounding tissue, probably due to the difference in consistency between brain tissues and beads. Their location was still perfectly visible as a round and well limited hole in the tissue. No beads were in the subarachnoidian space in any of the observed slides. The beads appeared round, and stained similar to basophile material (H&E staining), striated by the effect of the microtome knife, which is a sign of a hard material (these soft, hydrated beads become dehydrated during the tissue fixing process). There was no deformation, degradation or vacuolization of the beads over time (Fig. 1A). The striking point was the total lack of a foreign body reaction around the beads on H&E staining (Fig. 1B). Immunohistochemistry showed a very weak, non-specific, astrocytic reaction due to the implantation. There was no lymphocytic infiltration, nor demyelinisation. Neuronal markers showed healthy neurons and neuronal fibers in close proximity to the beads.

For the "100 mg/ml CM-BC2" group, rats received 400 µg of irinotecan per dose of beads. No clinical toxicity was observed. Nine animals were sacrificed according to the planned schedule at day 8 (n=3), month 3 (n=3) and month 6 (n=3). No brains showed macroscopic necrosis or macroscopic oedema (Fig. 2). For the 9 rats analyzed, beads were

observed in the striatum; in the middle of the striatum for 8 animals, and in the anterior part of the striatum for 1 animal. As for the other group, some beads were displaced from the implantation site during the slicing, again, with some tearing of the surrounding tissue on occasion, due to brain tissue and bead consistency differences. Their localization was still perfectly visible as a round and well limited hole in the tissue. The beads (or their localization) were in the striatum for all animals. In two animals, beads were observed in the subarachnoidian space due to a backflow. As before, the beads appear round, stained similarly to basophile material (H&E staining) and striated by the effect of the microtome knife. There was no deformation, degradation or vacuolization of the beads over time, at least until 6 months. The beads had the same appearance and stability as the unloaded control beads. At d8, a weak inflammatory reaction was observed around the beads, with macrophages and some lymphocytes. At 3 and 6 months, a moderate inflammatory reaction with some macrophages and siderophages was found (Fig. 3). There was a thin vacuolization of the brain tissue around the beads in one animal at month 3.

2. Combined effects of chemotherapy and radiotherapy

After the results obtained with the biocompatibility study, rats were randomized in five experimental groups. All non-treated rats died before d35, with a median and mean survival of 27 and 28.1 days (Fig. 4 and Table 2). Similar survival was observed in animals that received unloaded delivery beads, with all animals dying by day 31 (p = 0.16 versus control). Animals in group 3 (100 mg/ml CM-BC2) all died by day 41, with a median survival of 33 days. The survival was significantly different from untreated animals or those receiving unloaded control beads (respectively p=0.03 and p=0.003). When 100 mg/ml CM-BC2 was combined with radiotherapy (group 4), there was a significant increase in median survival (58 days) compared to unloaded beads (p=0.0006) or CM-BC2 alone (p=0.002). Combination of

100mg/ml CM-BC2 and WBI also improved survival with better median and mean survival times (58 and 60.8 days respectively) compared to the radiation therapy group (49.5 and 59.9 days respectively) although this result is not statistically significant. Survival curves for all groups were obtained by Kaplan-Meier Method (Fig. 4).

DISCUSSION

The purpose of this study was to assess the anti-tumor activity, in terms of survival benefit, of administration of drug-eluting beads in rats bearing a cerebrally-implanted syngeneic glioblastoma 9L. The potential added effect of radiotherapy was also evaluated. In terms of survival, the control groups (untreated and unloaded beads) median survival was as expected for this animal model in the laboratory (27 and 25 days, respectively), with a small standard error of the mean confirming the quality and reproducibility of the model. Fractionated radiotherapy significantly increased the survival, with a median survival of 49.5 days, in accordance with all the clinical literature (38, 39). There was no statistical difference between the untreated group and the unloaded-beads treated group. This result confirms the safety of the beads as we demonstrated it in the first part of the work. CM-BC2 (400 µg/rat) increased the median survival significantly, compared to the unloaded beads (p=0.003) (Fig. 5). This is consistent with the findings of Brinker *et al* (33) who also observed a statistically significant increase in survival for Irinotecan Drug Eluting Bead in the B4TCa rat glioma model. There are some additional data in literature concerning local administration of irinotecan. The most significant comes from the group of KS Bankiewicz, which developed CPT-11–nanoliposomes (40-42). It is interesting to point out that we obtain the same results as Bankiewicz with a lower dose of irinotecan This group demonstrated that, in the rat, convection enhanced delivery of free CPT-11 induces severe neurotoxicity at 400 µg/rat. When administrated in nanocapsules, however, there was no toxicity at any dose tested (60 µg to 1600 µg/rat). These data are in accordance with our results with 400 µg /rat and indicate that we could consider using a higher dose of irinotecan beads. In another rat glioma model (U87 xenograft), this group demonstrated that a single convection enhanced delivery of

nanoliposomal CPT-11 at 1600 µg/ rat improved median survival compared to blank nanoliposome or free drug.

Conventional treatments of human newly diagnosed brain tumors involve a systematical radiotherapeutic procedure (2, 38 and 39). We also evaluated the interest of the intratumoral administration of CM-BC2 associated to a fractionated radiation therapy. Irinotecan stabilizes a DNA-topoisomerase I cleaveable complex and acts as a good radiosensitizer (43). Combination of 100 mg/ml CM-BC2 and WBI further improved survival with better median and mean survival times (58 and 60.8 days respectively) compared to the WBI group (49.5 and 59.9 days respectively). One weakness of the study is that it would have benefitted if it were conducted with more rats in each group. Indeed, this result probably is not statistically significant because of the small number of animals per groups. However, it is clear that local chemotherapy using CM-BC2 gives an increase of 10 days more life expectancy to rats which were also irradiated.

The behaviour of our beads in rat brain was unknown even though this drug-device combination has been evaluated in a number of preclinical models that demonstrate the concept of high local delivery of the drug combined with lower systemic exposure. We also evaluate the biocompatibility and toxicity of these chemotherapy-loaded beads, after implantation into rat brain, by analyzing the clinical status of the rats, the macroscopic appearance of the brain, and the histological impact of the beads on the brain tissue. Other than this PVA hydrogel system, polyanhydride polymers (PCPP-SA) were prepared using the mix-melt method in one publication on local therapy in a rat glioma model (40). It is important to note that this polyanhydride polymer allows a slow release of the drug: 1% loaded polymer released 6.5% of drug over 200 hours, and 10% loaded polymer released 21% of drug over 200 hours. This release, slower than that obtained with the CM-BC2, could explain the differences in term of tolerance over different dose ranges. Otherwise, CM-BC2

(total dose 400 µg of irinotecan) were well tolerated with no clinical toxicity or histological toxicity. The lack of strong astrocytic reaction supports the supposition that the biocompatibility of the material in the brain is excellent. DC Bead microspheres are produced from a biocompatible polyvinyl alcohol (PVA) hydrogel that has been modified with sulphonate groups for the controlled loading and delivery of chemotherapeutic drugs (45-47). DC Bead occludes the blood flow to the target tissue and delivers a local and sustained dose of drug direct to the tumor. DC Bead received CE (European Conformity) mark approval in 2003 and can be loaded with doxorubicin or irinotecan amongst other agents (48). In this study, irinotecan-loaded beads were mixed with a solution of alginate. Alginate, a viscous polymer derived from brown algae, is an established compound in many biomedical and nutritional applications. Due to alginate's biocompatibility and simple gelation with divalent cations such as Ca^{2+}, it is widely used for cell immobilization and encapsulation. Mixing the alginate with the beads prior to intracerebral implantation was performed in order to minimize displacement of the beads. As we observed in biocompatibility studies, we have little back flow with this technique since only a few beads can be observed in the subarachnoid space in a few animals, and the reaction to the bead suspension demonstrates the excellent biocompatibility of both beads and carrier.

This preclinical evidence suggests that 100 mg/ml irinotecan-loaded beads are of considerable interest for patients with high-grade malignant brain tumors. The safety and efficacy for CM-BC2 in the rat model support a move to clinical studies in patients for a phase 1 trial.

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Antibodies anti-	Company	Clone	Dilution	Staining
CD3	Dako	Poly	100	T lymphocytes
GFAP*	Dako	Mono 6F2	900	Astrocytes
Neu N	Zymed	Mono A60	100	Neuronal bodies
Neurofilament	Monosan	Mono2F11	120	Axons
Synaptophysine	Biogenex	Mono Sup88	200	Neurons and their axons

a) GFAP: Glial fibrillary acidic protein

Table 1: Immunohistochemistry stains

GROUPS (n)	Range (days)	Median of survival (days)	Mean time of survival (days) ±SE	Mean ILT (%)	Long term survivors
Group « control » (n=8)	24-35	27.0	28.1 ±1.33	-	0
Group «unloaded beads » (n=7)	24-31	25.0	25.7±0.92	-	0
Group «WBI» (n=10)	33-120	49.5	59.9±2.85	133	2
Group «100 mg/ml CM-BC2 » (n=8)	27-41	33.0	33.6±1.85	31	0
Group « 100 mg/ml CM-BC2 + WBI » (n=8)	41-95	58.0	60.8±6.37	137	0

Table 2: Descriptive and statistical data from the survival study depending on groups of treatment. Group 1: group "control"; group 2: "unloaded beads"; group 3: "WBI (whole brain irradiation)"; group 4: "100 mg/ml CM-BC2" and group 5: "100 mg/ml CM-BC2 followed by WBI" of 18 Gy (3 x 6 Gy).

Increase in survival time (ILT) is calculated in comparison to the "unloaded beads" group (%). *SE* means "Standard Error".

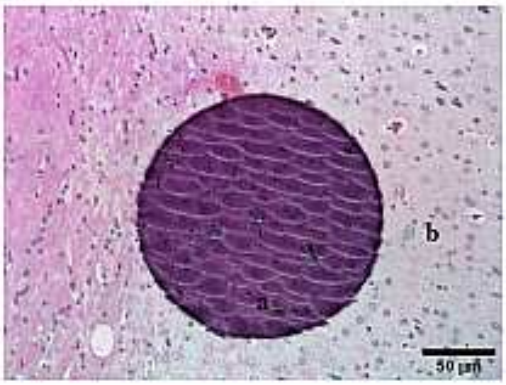

Fig. 1A: Unloaded beads in rat right striatum on month 6 (H&E x 20).

a: Unloaded beads. b: Respected neuronal nucleus

Fig. 1B: Unloaded beads in rat right striatum on month 6 (H&E x 2.5).

a: Unloaded beads. b: Striatum

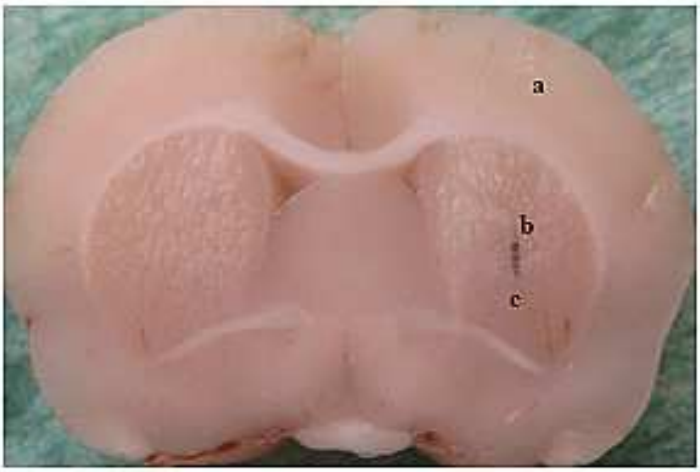

Fig. 2: Brain macroscopic examination of 100 mg/ml CM-BC2 on month 6. Rat's clinical state was good until the day of sacrifice. For every rat, macroscopic examination was normal without haemorrhage or oedema.

a: Cortex. b: striatum. c: 100mg/ml CM-BC2.

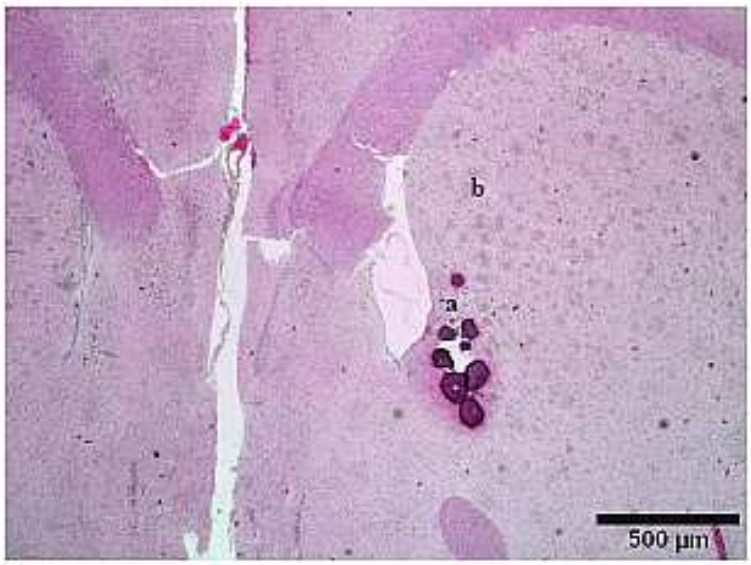

Fig. 3: 100 mg/ml CM-BC2 microscopic examination on M6 (H&E x 2.5).

a: 100mg/ml CM-BC2. b: Expected striatum.

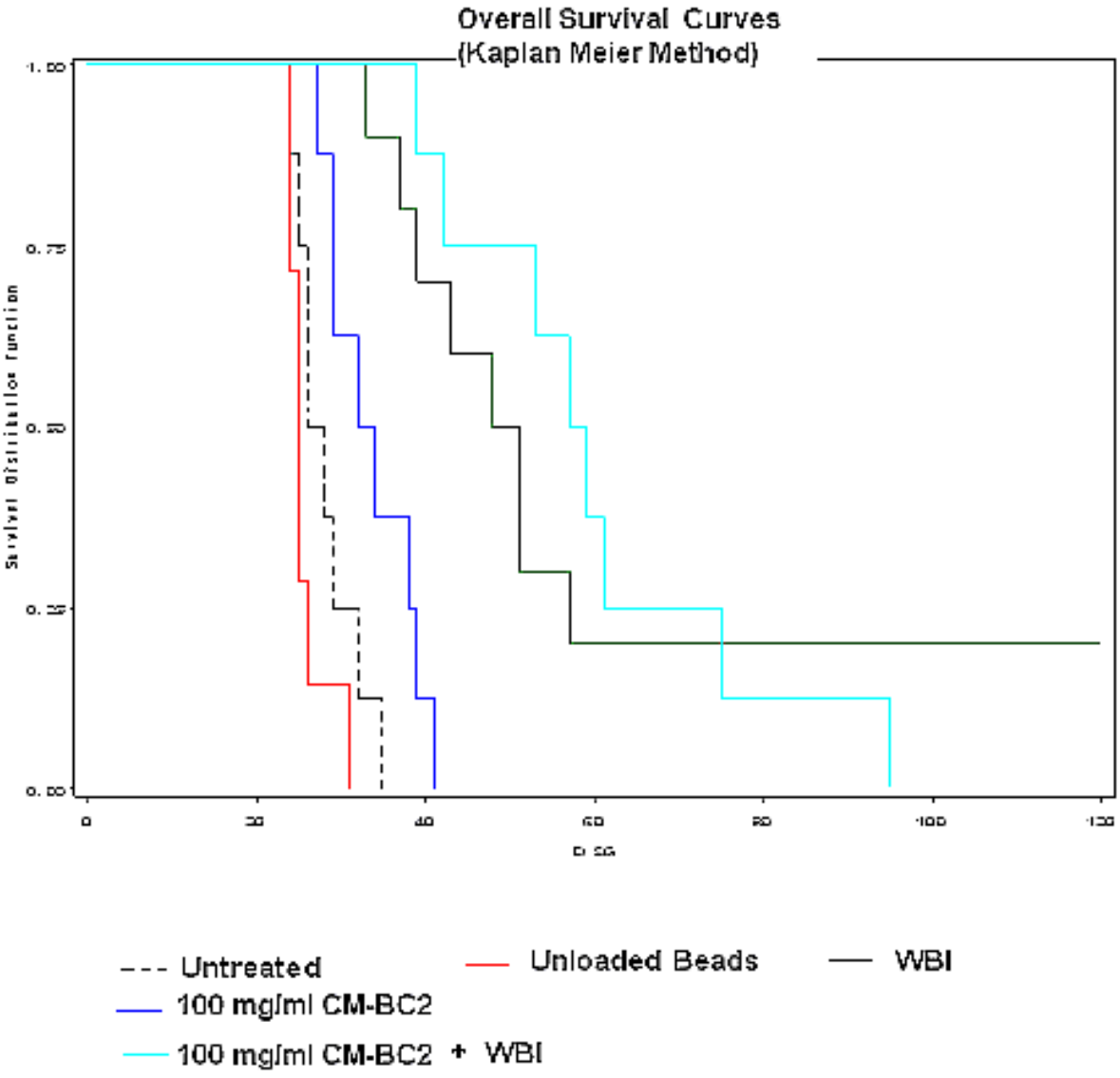

Fig. 4: Survival Curves obtained by Kaplan-Meier Method for the different groups of treatment.

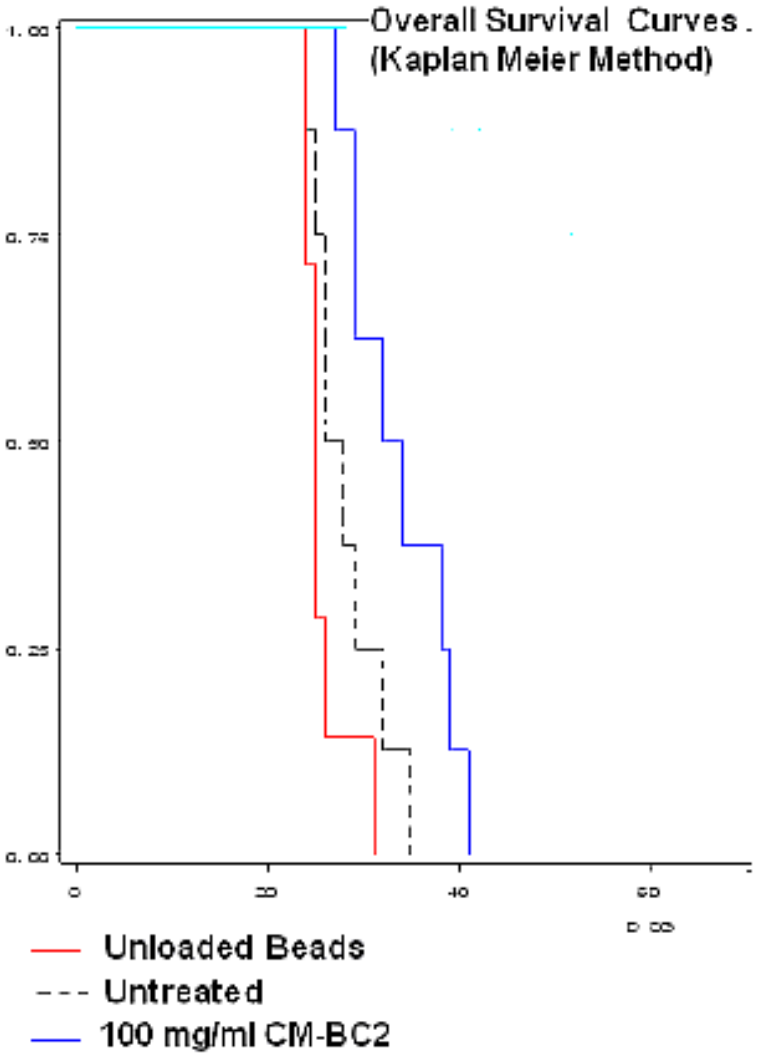

Fig. 5: Focus on the interesting results: survival curves obtained by Kaplan-Meier Method for the 100 mg/ml CM-BC2.

✓ **Abstract n°3**

- Local implantation of Irinotecan Drug Eluting Beads for 9L-rat glioma radiosensitization
Vinchon-Petit S, Jarnet D, Michalak S, Lewis A, Benoit J-P and Menei P.
15th Annual Scientific Meeting of the Society of Neuro-Oncology. November 18-21, 2010. Montreal. Canada.

SNO The Society for NeuroOncology

The 15th Annual Scientific Meeting of the Society for Neuro-Oncology
November 18-21, 2010 • Montreal, Canada

Control/Tracking Number: 10-A-133-SNO
Activity: Abstract

Local implantation of Irinotecan Drug Eluting Beads for 9L-rat glioma radiosensitization

Short Title:
Irinotecan Drug Eluting Beads

Author Block: Sandrine Vinchon-Petit[1], Delphine Jarnet[1], Sophie Michalak[2], Andrew Lewis[3], Jean-Pierre Benoît[4], Philippe Menei[2]
[1]Centre Paul Papin, [2]Centre Hospitalier Universitaire Angers, [3]Biocompatibles, [4]INSERM U646

Abstract:

Purpose: The main purpose of this study was to evaluate the safety and the efficacy of a local encapsulated chemotherapy (Irinotecan Drug Eluting Beads "CM-BC2"), usually used to treat hepatic tumors, in a 9L glioma model with or without irradiation.

Experimental Design: After brain injection of 100 mg/ml CM-BC2 (400 µg total dose of irinotecan) or unloaded beads, macroscopic and microscopic anomalies in the brain parenchyma were researched in 18 rats sacrificed on day 8, months 3 and 6. For therapeutic protocol, after tumor cells implantation, rats were treated locally as follows: (1) control group; (2) a group receiving unloaded beads, (3) a group whole-brain irradiation (WBI), (4) a group receiving 100 mg/ml CM-BC2 and (5) a group receiving 100 mg/ml CM-BC2 followed by a whole-brain irradiation (WBI).

Results: Both the unloaded beads and the 100 mg/ml CM-BC2 were well tolerated with no early deaths. Combination of 100 mg/ml CM-BC2 and WBI further improved survival with median and mean survival times of 58 and 60.8 days respectively. Notably, a higher median of survival was observed for the chemotherapy-radiotherapy protocol in comparison with the chemotherapy procedure (p=0.002).

Conclusions: 100mg/ml CM-BC2 showed minimal toxicity to normal brain parenchyma and had show some effect on survival in the 9L glioma model. The combination of chemotherapy and radiation therapy was well tolerated. This preclinical evidence suggests that combining radiation therapy with 100mg/ml irinotecan-loaded beads may benefit patients with high-grade malignant brain tumors and supports a move to clinical studies.

Disclosures & Release (Complete):

*****: My presentation will NOT include discussion of investigational or off-label use of a product.

*****: I agree my presentation will follow the guidelines stated above.

*****: I agree to statement above

Educational Objectives (Complete):

*** Educational Objective 1**

: 7. Use advances in pharmacology, experimental therapeutics, and radiobiology to improve future therapies for patients with CNS tumor.

b. Discussion

Nous avons limité notre volume d'injection à 20 µl. Nous ne pouvons injecter des quantités supérieures en intracrânien direct sans exposer les rats à des complications neurologiques. De plus, le volume tumoral traité est également restreint puisque la taille tumorale à J6 est estimée à environ 15 µl. Ces données peuvent peut-être expliquer nos résultats modestes en termes de radiosensibilisation.

- ✓ CM-BC1 ou microsphères de doxorubicine

La doxorubicine est une drogue d'une efficacité remarquable mais qui a déjà montré qu'elle pouvait être toxique. Les toxicités rapportées sont essentiellement neurologiques et cardiaques lorsqu'elle est utilisée par voie systémique.

C'est d'ailleurs pour une meilleure tolérance que des formes liposomales ont vu le jour. Lesniak et al. ont testé l'une d'entre elles, un polymère polyanhydride (PCPP-SA) implanté localement pour s'affranchir de toute toxicité systémique. Tout comme nous, ils ont expérimenté des implants présentant différentes concentrations de doxorubicine et tout comme nous, ce sont les polymères les moins chargés qui se sont révélé les mieux tolérés. Ils ont également retrouvé des hémorragies et de la nécrose dans les cerveaux des rats appartenant aux groupes ayant reçu des doses de doxorubicine plus élevée (Photo 11). Comme dans notre étude, la réponse étant malheureusement corrélée à la dose, les implants les moins chargés sont moins efficaces.

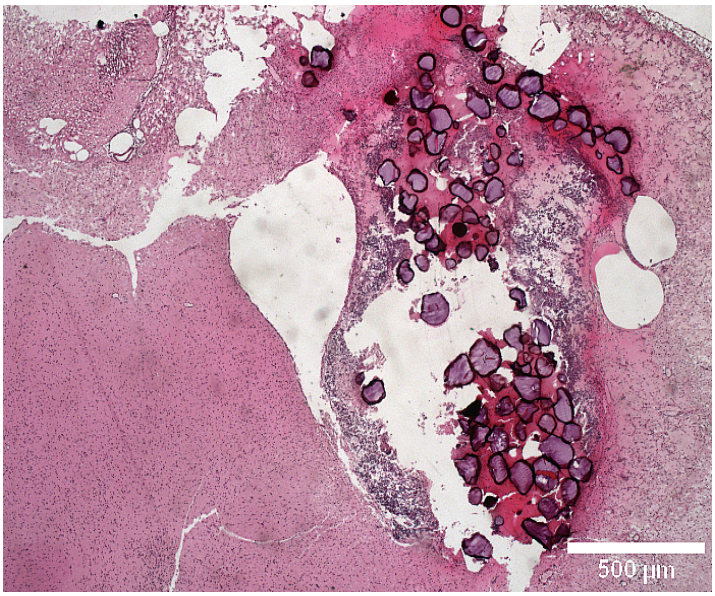

Photo 11: Nécrose cérébrale observée chez nos rats traités par les CM-BC1 à la concentration de 10 mg/ml.

Nos rats traités avec les CM-BC1 à 1 mg/ml de doxorubicine recevaient donc 4 µg de doxorubicine après formulation des solutions mixées à l'alginate du fait du phénomène de dilution. En effet, lors de la reconstitution de la solution de microsphères avant injection, à 1 ml de microsphères, sont ajoutés 2 ml d'eau stérile et 3 ml d'alginate. Lesniak a démontré dans des travaux plus anciens qu'*in vitro*, à la concentration de 50 ng/ml de doxorubicine libre, au moins 97,5% des cellules 9L étaient inhibées à J5 (81). Ces données sont en accord avec nos résultats. L'implantation locale de CM-BC1 à 1 mg/ml augmente significativement la survie des rats comparés à ceux traités par microsphères blanches (p<0,03). Ainsi, le fait de ne pas obtenir de différence significative entre l'association radio-chimiothérapie et la radiothérapie seule n'est pas lié à l'absence d'efficacité des CM-BC1 mais à une absence de radiosensibilisation.

Ainsi, les CM-BC1 n'étaient pas faciles à manier du fait de leur toxicité potentielle lié à la doxorubicine et notre objectif, qui est d'améliorer la radiosensibilité du gliome n'était pas atteint. Nous avons alors focalisé nos efforts sur les microsphères d'irinotécan ou CM-BC2.

- ✓ CM-BC2 ou microsphères d'irinotécan

Les microsphères blanches et les microsphères CM-BC2 étaient quant à elles très bien tolérées sans réaction neurologique histologique ou clinique. De plus, le groupe traité par CM-BC2 seul présentait une survie très significativement augmentée par rapport au groupe traité par microsphères blanches (p=0,003). Ces résultats sont intéressants si l'on songe à ce qui se prescrit en pratique clinique. L'association «bévacizumab + irinotécan» n'est pas indiqué dans les glioblastomes nouvellement diagnostiqués mais lors de la récidive, une fois la radiothérapie déjà réalisée. Même si notre objectif initial était l'étude de la radiosensibilité des CM-BC2, ces données sont néanmoins encourageantes compte-tenu de leur profil de tolérance.

Une étude pilote a été menée par Brinker et al avec les mêmes microsphères de doxorubicine et d'irinotecan sur un modèle B4TCa de gliome du rat (82). Alors que les 2 types de microsphères étaient efficaces sur la tumeur, les microsphères de doxorubicine étaient beaucoup trop toxiques et la méthode d'injection des microsphères n'était pas transposable en clinique.

Pour notre part, nous avons utilisé une formulation contenant de l'alginate. Son avantage était de modifier la viscosité de la solution et de faciliter son injection en évitant les reflux vers la surface du cortex cérébral. Nous avons également injecté des volumes plus importants sans léser le parenchyme cérébral des rats injectés.

Ces différents éléments nous ont permis de prétendre à la mise en place d'une étude de phase I et une demande d'autorisation est en cours auprès de l'EMA (European Medicines Agency).

Si cette donnée est encourageante, les autres résultats obtenus ne constituent pas une finalité. Pour ces systèmes polymères, l´efficacité de l'administration directe de la chimiothérapie est très certainement réduite par une diffusion limitée à partir des implants aboutissant à un index thérapeutique élevé mais dans un volume tissulaire tumoral limité. De plus, plus les connaissances sur les modifications biomoléculaires présentées par les gliomes s'affinent, plus il est frustrant de ne pouvoir intégrer ces données dans la prise en charge thérapeutique active des patients comme c'est le cas par exemple pourles tumeurs gliales de bas rade dont la chimiosensibilité dépend de la présence de la délétion 1p 19q. Les outils thérapeutiques envisagés se heurtent encore largement au caractère échappatoire des gliomes, qui peut être associé à l'expression élevée de protéines de survie telles que la protéine Bcl-2, à l'expression de transporteurs d'efflux participant au phénomène de MDR (résistance *"multidrug"*), ou à un coefficient de prolifération abaissé pouvant notamment entraîner une insensibilité à des doses d'irradiation inférieures à 60 grays.

2.4. Les nanocapsules lipidiques dans la prise en charge locale des tumeurs gliales 9L du rat.

a. Présentation du travail

L'utilisation de la CED évite la survenue d'un reflux le long du trajet favorisé par l'aiguille et ce de par la pression positive permanente et continue qu'elle génère. Cette notion prend toute son importance pour l'injection de particules nanométriques encore plus soumises aux contraintes de pression de par leur petite taille que les macromolécules. De nombreuses molécules, habituellement utilisées en oncologie clinique par voie systémique, ont déjà été introduites en injection locale par CED. Parmi elles, le témozolomide (83), le topotécan (84), le cisplatine (85). Trois études ont été réalisées avec du paclitaxel libre. L'étude de Pöpperl *et al* étudiait par FET (O-(2-[18F] fluoroéthyl)-L Tyrosine) PET (positron emission tomography) les effets d'une CED de paclitaxel chez des patients atteints de glioblastome récurrent (86). Deux patients étaient décédés de complications locales 4 et 5 mois après le traitement. Six patients sur 8 présentaient une stabilité temporaire de leur maladie suivie d'une progression mais située à distance de la CED pour 5 patients sur 6 dans les 3 à 13 mois suivant la CED. Un patient était toujours en bon état neurologique à la fin de leur étude soit 10 mois après la CED. La 2ème étude, l'étude de Mardor *et al.* , était également focalisée sur la validation d'une méthode d'imagerie pour le suivi de patients traités par CED de paclitaxel (87). La DWIRM (diffusion-weighted IRM) permettait de suivre les modifications parenchymateuses cérébrales imposées par la CED de paclitaxel. Différents types de réponse au traitement avaient été observés sans conclusion véritable sur l'efficacité de ce type de traitement. La 3ème et dernière étude, celle de Lidar *et al.* , était une étude de phase I/II sur 15 patients porteurs de glioblastome récurrent (88). Le taux de réponse était de 73%. L'absence de tumeur était confirmée par réintervention chirurgicale chez cinq patients. Ainsi, cinq patients sur 15 présentaient une réponse complète et six une réponse partielle.

Outre son activité cytotoxique directe contre les cellules gliales, le paclitaxel constitue un bon radiosensibilisant. Il s'associe à la ß-tubuline, principal composant des microtubules et favorise leur polymérisation. Une fois qu'ils sont stabilisés, il empêche ensuite leur

dépolymérisation. La cellule est alors bloquée en phase G2M, phase la plus radiosensible du cycle cellulaire (Laboratoires Bristol-Myers Squibb 1994). Cependant, en solution, il présente trois limites principales. Premièrement, il pénètre faiblement la BHE et atteint difficilement le système nerveux central (89). Secondairement, il possède un effet dose et temps-dépendant (90) et troisièmement, il est le substrat de la P-gp et d'autres transporteurs d'efflux qui empêchent son action cytotoxique (91) (Figure 9). Ces divers éléments ont justifié l'étude de sa vectorisation.

Il a été établi que les LNC chargées en paclitaxel sont plus efficaces que le paclitaxel en augmentant la mort des cellules cancéreuses *in vitro* et en réduisant l'expansion tumorale des gliomes implantées en sous-cutané *in vivo* (32). Ces données n'ont jamais été confirmées *in vivo* sur des tumeurs gliales implantées en intra-cérébral. Le but de cette étude était donc de travailler dans les conditions rencontrées en pratique clinique, où la tumeur cérébrale interagit avec son micro-environnement.

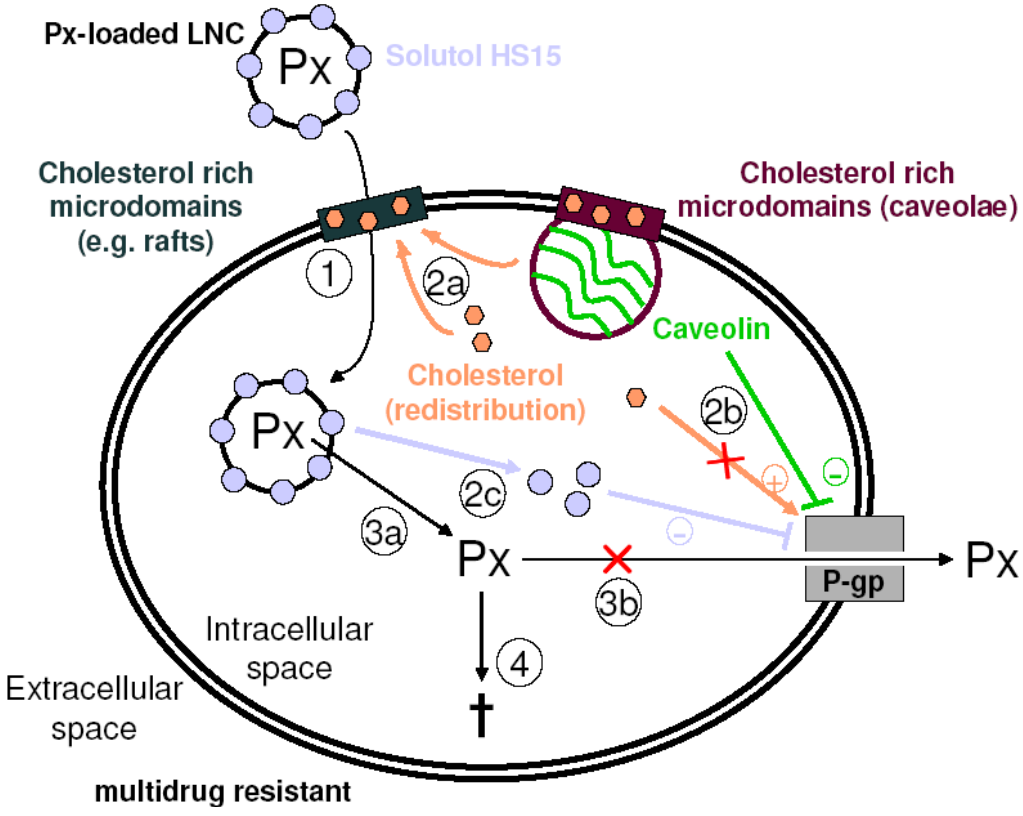

Figure 9: Illutrations des propriétés des LNC, notamment le phénomène anti-MDR.

✓ **Article n° 5**

- In vivo evaluation of intracellular drug-nanocarriers infused into intracranial tumours by convection-enhanced delivery: distribution and radiosensitisation efficacy.
Vinchon-Petit S, Jarnet D, Paillard A, Benoit JP, Garcion E, Menei P. J Neurooncol. 2010 Apr; 97(2):195-205.

J Neurooncol
DOI 10.1007/s11060-009-0012-4

LABORATORY INVESTIGATION - HUMAN/ANIMAL TISSUE

In vivo evaluation of intracellular drug-nanocarriers infused into intracranial tumours by convection-enhanced delivery: distribution and radiosensitisation efficacy

Sandrine Vinchon-Petit · Delphine Jarnet · Archibald Paillard · Jean-Pierre Benoit · Emmanuel Garcion · Philippe Menei

Received: 26 May 2009 / Accepted: 14 September 2009

Abstract The objective of the present study was to investigate the interest of convection-enhanced delivery (CED) for the administration of a nanocarrier-based radiosensitizing chemotherapy in the rat brain. Pursuing on newly developed lipid nanocapsules (LNC) that can be internalised within brain tumour cells, we studied their intracerebral distribution when labelled with fluorescent Nile red (NR). As paclitaxel (Px) represents an interesting radiosensitiser, we also evaluated the potential radiosensitising effects of Px-loaded LNC administered through CED in the 9L intracranial rat glioblastoma model. The distribution study demonstrated that CED injection of NR-loaded LNC (NR-LNC) improved significantly the volume of distribution of NR when matched with simple injection (by about 150 fold). It also reveals that the LNC perfusion of a whole tumour forming area inside the CNS (6 days after implantation of 10^3 9L cells) is achievable through CED injection, whilst preserving the ability of LNC to reach the intracellular space of encountered tumour cells. Having established an animal model of encephalic irradiation close to the clinic (18 Gray in three fractions of six Gray at days 8, 11 and 14 after 9L cell implantation) we proved the feasibility of the combination of CED for the administration of drug-loaded LNC with external beam therapy. Although a single CED injection of Px-LNC at low Px dose (375 µg/kg of bodyweight) gave the best median survival (twice that of untreated controls), it underlines the need for optimisation. Hence, the possibility of grafting recognition moieties onto the LNC surface combined to their biocompatibility must be beneficial.

Keywords Nanotechnology · Nanomedicine · Nanoparticle uptake · Brain drug delivery · Paclitaxel bioavailability · Radiochemotherapy

S. Vinchon-Petit · A. Paillard · J.-P. Benoit · E. Garcion (✉) · P. Menei
Inserm U646, Université d'Angers, 10 rue André Boquel, 49100 Angers, France
e-mail: emmanuel.garcion@univ-angers.fr

P. Menei
Département de Neurochirurgie, CHU, 4 rue Larrey, 49033 Angers, France

D. Jarnet
Centre Régional de Lutte Contre le Cancer, Centre Paul Papin, 2 rue Moll, 49933 Angers, France

Introduction

The treatment of glioblastoma, a primary malignant tumour of the brain, remains one of the most challenging cancer problems, as no curative treatment has yet been found [1]. Conventional therapeutic procedures focus on surgical resection combined with adjuvant radiochemotherapy. However, despite constant refinements in these techniques, tumour recurrences are common and patients continue to die. So far, the best survival results have been obtained by the use of temozolomide combined with external beam radiotherapy [2]. Hence, most currently-available antiglioma therapies have less-than-optimal usefulness, mainly owing to delivery problems to the tumour, including systemic toxicity and crossing the blood–brain barrier (BBB) [3]. Although curing glioblastoma will likely depend on the discovery of an anticancer entity that will destroy all cancer cells or make them sensitive to destruction, new therapy will also require innovative methods of drug delivery to reach effective doses on infiltrative cancer cells

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and to reduce side effects. Thus, direct administration into the brain parenchyma of locally implanted drug delivery systems emerges as an interesting alternative to overcome these concerns [4]. It may also present the advantage of preventing drug degradation before it would have reached its expected molecular target in the brain.

Among innovative domains, nanotechnology may provide the opportunity to shape new brain-implantable nano-objects for improved efficacy, specificity and biological safety [5–7]. As such, we have recently developed lipid nanocapsules (LNC) that were shown to increase anticancer hydrophobic drug efficacy while also targeting glioma cell intracellular compartments and improving drug bioavailability through inhibition of multidrug resistance phenomena [8, 9].

Pursuing this work and by considering drug bioavailability issues together with conventional treatments of human brain tumours that systematically involve a radiotherapeutic procedure, the objective of the present study was to evaluate the interest of the intratumoural administration of a new nanomedicine-based radiosensitising chemotherapy. Firstly, since convection-enhanced delivery (CED), which is a novel regional method that allows the distribution of substances throughout the interstitium via positive-pressure infusion, has been shown to enhance the volume of distribution (Vd) of administered compounds [10], we investigated its benefit and relevance for the administration of LNC in the rat brain. Secondly, as Paclitaxel (Px) represents a remarkable radiosensitiser, acting on the G2-M phase of cell division [11], we evaluated the potential radiosensitising effects of Px-loaded LNC administered through CED in the 9L intracranial Fischer rat glioblastoma model.

Materials and methods

Animals

Seventy-four syngeneic F344 Fischer female rats (Charles River, Cléon, France) weighing 150–200 g were used for this study. They were kept in standard animal facilities with free access to food and water. All experiments were conducted under good experimental practices according to the European Commission and to the French Ministry of Agriculture regulations.

Tumour model

9L glioma cells (European Collection of Cell Cultures, Salisbury, UK) were grown at 5% CO_2 and 37°C in Dulbecco modified Eagle medium containing glucose, L-glutamine, 10% foetal bovine serum (Biowhittaker, Verviers, Belgium) and 1% antibiotic and antimycotic solution (Sigma, Saint-Quentin Fallavier, France). For tumour induction, 9L cells were trypsinised by trypsine/EDTA (Sigma), washed and resuspended at 10^3 cells in 10 µl Eagle minimum essential medium (Biowhittaker) before surgical stereotaxic implantation.

Formulation and characterization of the nanocarriers

Fifty nm-diameter LNC (Fig. 1A) were synthesized by using a previously described phase inversion-based process that follows the formation of an oil/water microemulsion containing an oily fatty phase (triglycerides: Labrafac®), a non-ionic hydrophilic surfactant (polyethylene glycol hydroxystearate: Solutol® HS15) and a lipophilic surfactant (lecithins: Lipoïd®) [9]. Briefly, Solutol® HS15 (external shell), Lipoïd® (internal shell), Labrafac® (lipid core), NaCl and water (846, 75, 1029, 89, 2975 mg, respectively) were mixed and heated under magnetic stirring up to 85°C. Three cycles of progressive heating and cooling in between 85 and 60°C were then carried out and followed by an irreversible shock induced by dilution with 12.5 ml of 0°C deionised water added to the mixture at 70°C.

For fluorescent labelling of LNC, Nile red (NR, Sigma) was used as described previously [12]. Briefly, NR was dissolved in acetone at 1‰ (w/w) and resulting solution incorporated in the triglyceride phase (Labrafac®) at 1/10 (w/w) before formulation. A BD FACSAria™ fluorescent-activated particle sorter and the BD FACSDiva™ software (BD Biosciences, Le Pont de Claix, France) were used to determine the efficiency of the LNC labelling.

For the formulation of Px-loaded LNC, 20 mg of Px was first solubilised in a solution of 206 mg ethanol, 206 mg dichloromethane and 10 mg cholesterol. Solutol® HS15, Lipoïd®, Labrafac®, NaCl and water were then added to this solution and the formulation of Px-loaded LNC performed according to the above procedure with the evaporation of dichloromethane and ethanol during the process. The Px encapsulation rate was determined after the separation of LNC from the supernatant using Centrisart C30 microcentrifuge filters (Sartorius, Goettingen, Germany). The measurement of the concentration was carried out in triplicate in the supernatant by HPLC and lead to an encapsulation rate of 93.0 ± 3.1% according to the initial amount of drug added.

LNC were analysed for size and charge distribution using a Malvern Zetasizer® Nano Serie DTS 1060 (Malvern Instruments S.A., Worcestershire, U.K.). They were diluted 1/100 (v/v) in deionised water in order to assure a convenient scatter intensity on the detector. All

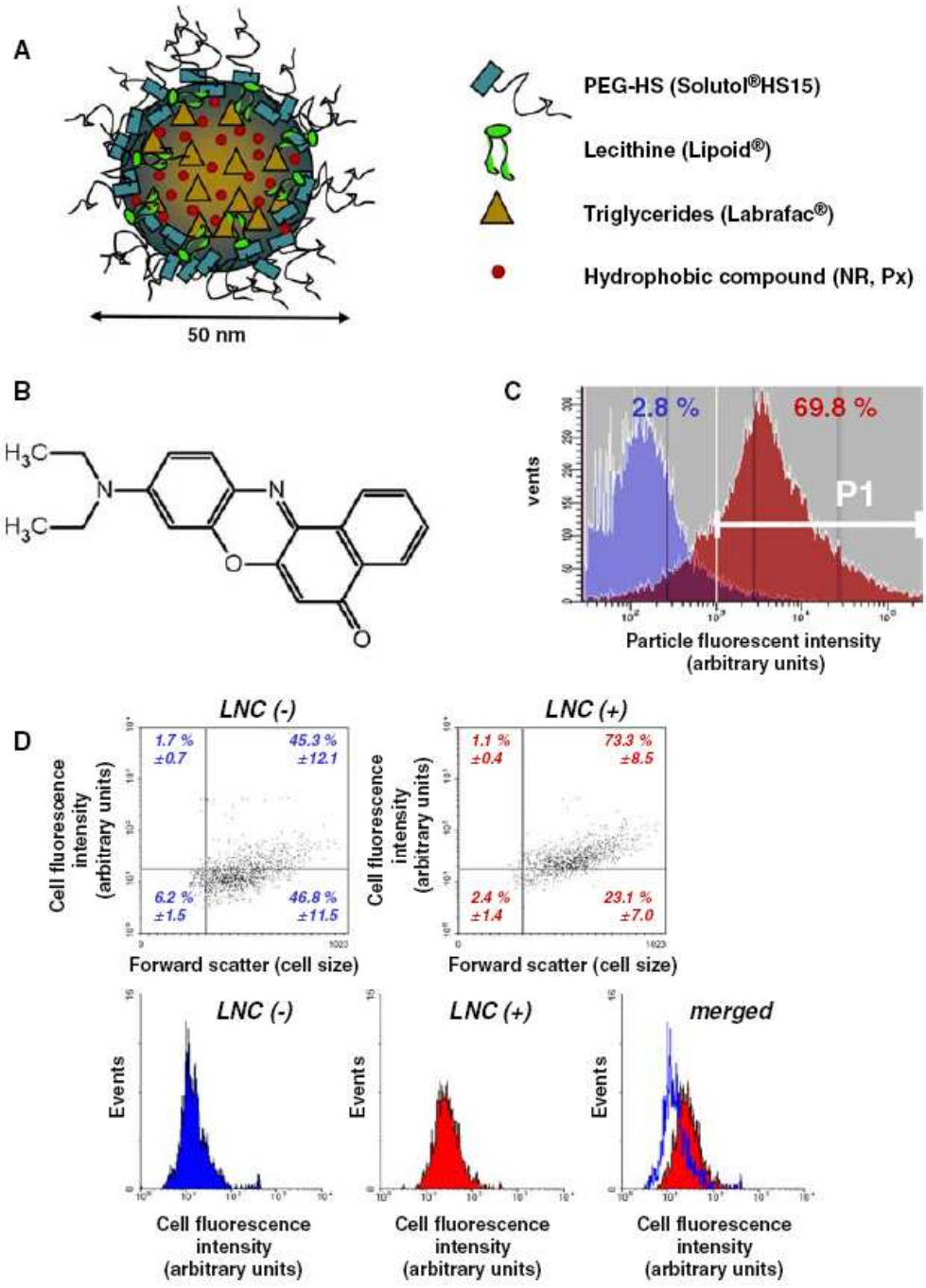

Fig. 1 In vitro characterisation of the uptake of NR-stained LNC by 9L glioma cells. **A** Schematic representation of drug-loaded LNC. **B** Molecular structure of NR. **C** Fluorescent-activated particle sorting of classical blank LNC (2.8% in P1) and NR-stained LNC (69.8% in P1). **D** Flow cytometry analysis of NR uptake by 9L cells after 2 h of incubation (LNC (−), control without LNC; LNC (+), cell treated with NR-LNC)

Table 1 LNC physicochemical characteristics

LNC type	Size (nm)	Zeta potential (mV)
Blank-LNC	50.8 ± 0.1	−4.02 ± 0.71
NR-LNC	51.2 ± 2.9	−2.51 ± 0.48
Px-LNC	53.9 ± 0.6	−3.95 ± 0.51

nanocarriers (loaded or not) presented a monomodal particle size distribution around 50 nm with a narrow distribution (polydispersity index < 0.3) (Table 1).

Cellular analysis of Nile red-loaded LNC (NR-LNC) uptake by flow cytometry

A BD FACSCalibur fluorescent-activated flow cytometer and the BD CellQuest software (BD biosciences) were used as previously described [12]. To discriminate between cell-association and actual internalisation, the removal of attached LNC was carried out by using a solution of acetic acid and NaCl. Extracellular fluorescence was quenched by the addition of Trypan Blue, the non-quenched fraction thus representing internalised NR-LNC [13].

Confocal laser scanning microscopy

Confocal microscopy was performed as previously described [12] and images acquired by using an Olympus Fluoview FU 300 confocal microscope imaging system (Paris, France).

Preparation of Px solutions

Free Px solutions were prepared from a commercial 6 mg/ml solution (Taxol®, Bristol-board Myers Squibb) to obtain a final concentration of 1.25 mg/ml (identical to the one in Px-LNC).

Surgical procedures

Rats were anaesthetised IP with 10 mg/kg xylazine (Rompun) and 50 mg/kg ketamine (Clorketam) before being placed in a Kopf stereotaxic frame (Harvard Apparatus, Les Ulis, France). After shaving, disinfection and sagittal incision of the cranial skin, a burr hole was made in the skull at 0.5 mm anterior and 3 mm lateral from the Bregma using a small drill. Suspensions were then injected at 5 mm below the dura in the right striatum according to the Paxinos atlas. The needle was afterwards removed cautiously before the wound was sutured.

CED

CED was carried out using a final volume of 60 µl. Briefly, the system consisted of a 10 µl—32 gauges (32G) Hamilton® syringe (Harvard Apparatus) or a 500 µl—29G Omnican® 50 syringe (Braun, Boulogne, France) connected by a polyethylene Co-Ex™ PEPVC tube to a Hamilton® syringe of 100 µl mounted onto a PHD 2000 programmable infusion pump (all from Harvard Apparatus). Connections were secured with 2-hydroxyethylmethacrylate and the system was checked for leak before use. A 0.5 µl/min constant infusion rate was applied.

Study design

LNC distribution study

One of the major advantages of CED on simple injection resides in improving volumes of distribution, as a result of which it is also possible to increase the volume of infusion without affecting the integrity of brain parenchyma and the efficacy of infusion. Thus, considering the theoretical maximum volume that can be infused through each technique the final volumes of infusion used in the present study were different between the bolus- [14] and CED-injected animals [15]. Although most of a rat striatum can be perfuse with CED with 20 µl macromolecules in solution such as albumin [16], convection, diffusion and distribution of larger nanoparticle entities in suspension are expected to differ [17]. Hence, several CED studies have used higher infusion volumes for liposomes [18] or prolonged CED with dextran [19]. Thus, together with consideration of the drug dose that can be reached in the efficacy study, a volume of infusion of 60 µl on 2 h was chosen for CED injection of 50 nm LNC. Rats were randomly assigned to four groups with five to six rats per group. Two groups received a simple intracranial stereotaxic injection (SI) of fluorescent LNC (10 µl) with either a 26G needle (SI-26G) or a 32G needle (SI-29G); two groups received a CED injection of 60 µl of fluorescent LNC with either a 29G needle (CED-29G) or a 32G needle (CED-32G). All rats were killed by CO_2 inhalation on Day 1. The brains were surgically removed, snap-frozen in liquid nitrogen-chilled isopentane and stored at −80°C. Frontal cryosections (14 µm) throughout the whole injection site were performed using a Cryocut 3000 (Leica, Rueil-Malmaison, France). The resulting slides were kept at −20°C before processing. They were then allowed to dry for 30 min at room temperature, fixed in 4% paraformaldehyde and stained with 1/1000 DAPI (Sigma) in PBS for 10 min. Finally, they were washed 3 times with PBS before mounting in 1/1 (v/v) PBS/Dako Cytomation fluorescent mounting medium (Trappes, France). All slides were examined under an Axioskop-2 Zeiss fluorescence microscope (Le Peck, France). Images were acquired through a Photometrics CoolSNAP ES camera equipped with a QImaging CRI Micro Color 2 RGB Liquid Crystal filter and by using the MetaVue™ imaging system (all from Roper Scientific, Evry, France). The images were then analysed using the Image J software (NIH, USA). The apparent volume of distribution (Vd) for each rat according to the type of injection was calculated after extrapolation of the maximum surface area labelled by NR on overall brain tissue slices. The length (L) and the width (l) of the fluorescent zone were automatically measured after manual contouring and the Vd was estimated according to the mathematical ellipsoid formula: $\pi/6 \times L \times l^2$. The statistical significance between groups was determined by ANOVA and Kruskal–Wallis tests for mean comparison by pairs.

Efficacy study

Rats were randomly assigned to five groups and implanted with 9L tumour cells. Group A was the untreated group. Group B was the 'radiotherapy only' group. Groups C, D and E were the 'radio-chemotherapy' groups in which 60 µl of chemotherapy was delivered by CED on day 6 after tumour cell implantation. Rats from Group C received free Px at the dose of 375 µg/kg of bodyweight; rats from Group D received Px-loaded LNC (same Px dose) and rats

from Group E received blank LNC. Anaesthesias was maintained in each group. Rats were irradiated in the Radiotherapy Department using a Saturn 41 linear accelerator. Under deep anaesthesia by using 4.5% isoflurane for 2 min, then at 2% combined with 3 l/min O_2, the rats were installed in ventral decubitus with head and body lined up for the lasers. The brain irradiation was delivered by two photon beams, parallel and opposed, at 6MV-energy, with DSP 100 and a flow of 4 Gy/min. The size of the field was 15 × 15 cm. The posterior limit of the field corresponded to the line passing between the posterior part of the two ears. A bolus of 1.5 cm was laid out on the surface of the rat's head in order to homogenise the amount received on the brain surface. 18 Gy in three fractions of 6 Gy were delivered. The irradiation began in the absence of scarring trouble (e.g. abscess, contusion) and fine general health. After irradiation, the animals were replaced in their cage under the same conditions. They were examined daily. Rats too weak to feed and to stand, thus presenting clinical signs of tumour progression, were killed and their brain removed for analysis. Medians survival rates were calculated from the day of the tumour implantation. The survival curves were obtained by the Kaplan–Meier method. The comparison of these curves used the Log-rank or Mantel–Cox test. Rats that survived up to 106 days were regarded as long-term survivors.

Results

LNC monitoring in 9L glioma cells

Among drug delivery systems, nanocarriers may offer the opportunity to be internalised in non-phagocytic eukaryotic cells as a result of their small size. To explore such behaviour on 9L rat glioma cells, LNC (Fig. 1A) were loaded with the fluorescent hydrophobic compound NR (Fig. 1B). Fluorescent-activated particle sorting at 488 nm showed a noticeable increase in the Gaussian distribution of LNC numbers around a high fluorescent intensity median after NR incorporation, thus demonstrating an efficient LNC labelling (Fig. 1C). NR-LNC uptake by 9L glioma cells was then followed by fluorescent-activated flow cytometry (Fig. 1D). As size exclusion followed by HPLC quantification demonstrated that 96% of the NR used was encapsulated within the LNC and since free NR cannot be transferred on its own to the 9L cells during the 2 h incubation period [12], NR uptake by 9L glioma cells, when using NR-LNC, can be quoted as actual LNC uptake. An examination of cells before [LNC (−)] and after 2 h incubation with NR-LNC [LNC (+)] revealed a marked increase in cell fluorescence intensity, thus demonstrating LNC uptake (Fig. 1D). These data were confirmed by confocal experiments showing strong NR labelling in 9L cell perinuclear and cytoplasmic areas which avoided the nucleus (Fig. 2).

On the use of CED for the administration of LNC within the CNS

Classical stereotaxic injections of drugs within the brain are often limited by the volume injected as well as by a poor tissue penetration, thus resulting in small Vd and weak local doses [20]. As CED has been shown to improve the delivery of agents in the CNS to large volumes of tissue [10], we evaluated the interest of this minimally invasive surgical technique for the intracerebral administration of LNC. Dealing with the possibility to increase infusion volumes, we compared in healthy animals [normal brain tissue] the use of classical simple injection (SI) with 10 µl injected in 5 min to that of CED with 60 µl injected in 2 h. We also put side by side the use of two distinct needles: 26G and 32G for SI and 29G and 32G for CED. On the basis of our work on cell culture demonstrating that the hydrophobic dye NR can be considered as a witness of LNC distribution, we used NR-LNC for intracerebral

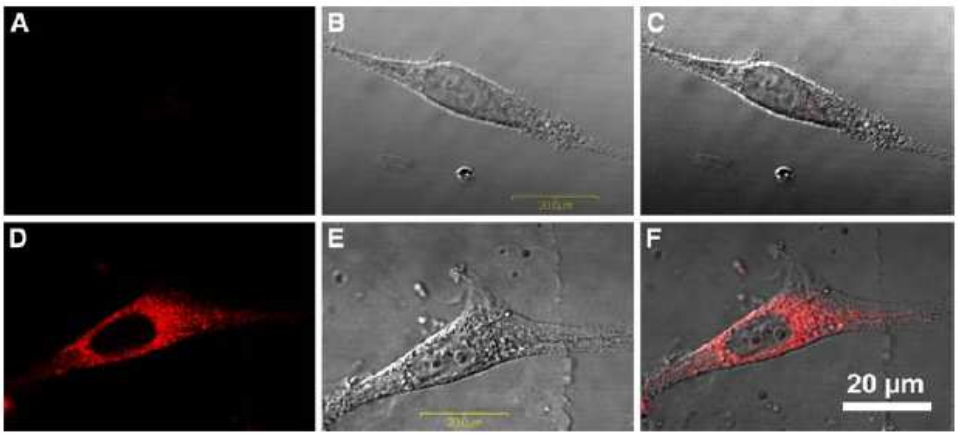

Fig. 2 Confocal laser scanning microscopy images of 9L cells after 2 h incubation with NR-LNC (**A**–**C** control cell; **D**–**F** treated cell; **A**, **D** Nile red fluorescence; **B**, **E** Nomarsky; **C**, **F** merged pictures)

implantation and NR fluorescence as a proportional indicator of areas occupied by LNC in brain slices. For consistency, analyses were carried out 24 h after the injection of NR-LNC (a time sufficient for potential interactions of LNC with CNS cells and short enough to give a representative idea of the infusion). With SI-26G, fluorescence microscopy analysis revealed a back leakage in the cortex and the corpus callosum along the needle path (Fig. 3). Of the five rats tested, two presented a small NR-labelled area around the injection site whereas no fluorescence was detected in the striata of the three others. Interestingly, with SI-32G the outflow was prevented (Fig. 3). In rats that were injected with CED-29G or CED-32G a satisfactory tolerance was proven with no post-injection neurological deficit or deterioration of the general health. Microscope slide analysis demonstrated that CED significantly improved the dispersal of NR throughout the brain parenchyma in comparison to classical stereotaxic injection (Fig. 3). Brain slices from CED injected rats also presented the characteristic gradients of convection and diffusion and a better NR homogeneity around the injection site (Fig. 3). No major differences were found between CED-29G or CED-32G, except for the presence of a weak fluorescence at the cortex surface when using a 29G needle, which was not however combined with any staining along the needle path (Fig. 3). To quantify NR-LNC distribution within the brain parenchyma, computer slide analysis was performed. Our data presented on Fig. 4 demonstrated a strong improvement of the Vd when using CED versus classical direct administration. On average, CED enhanced the Vd

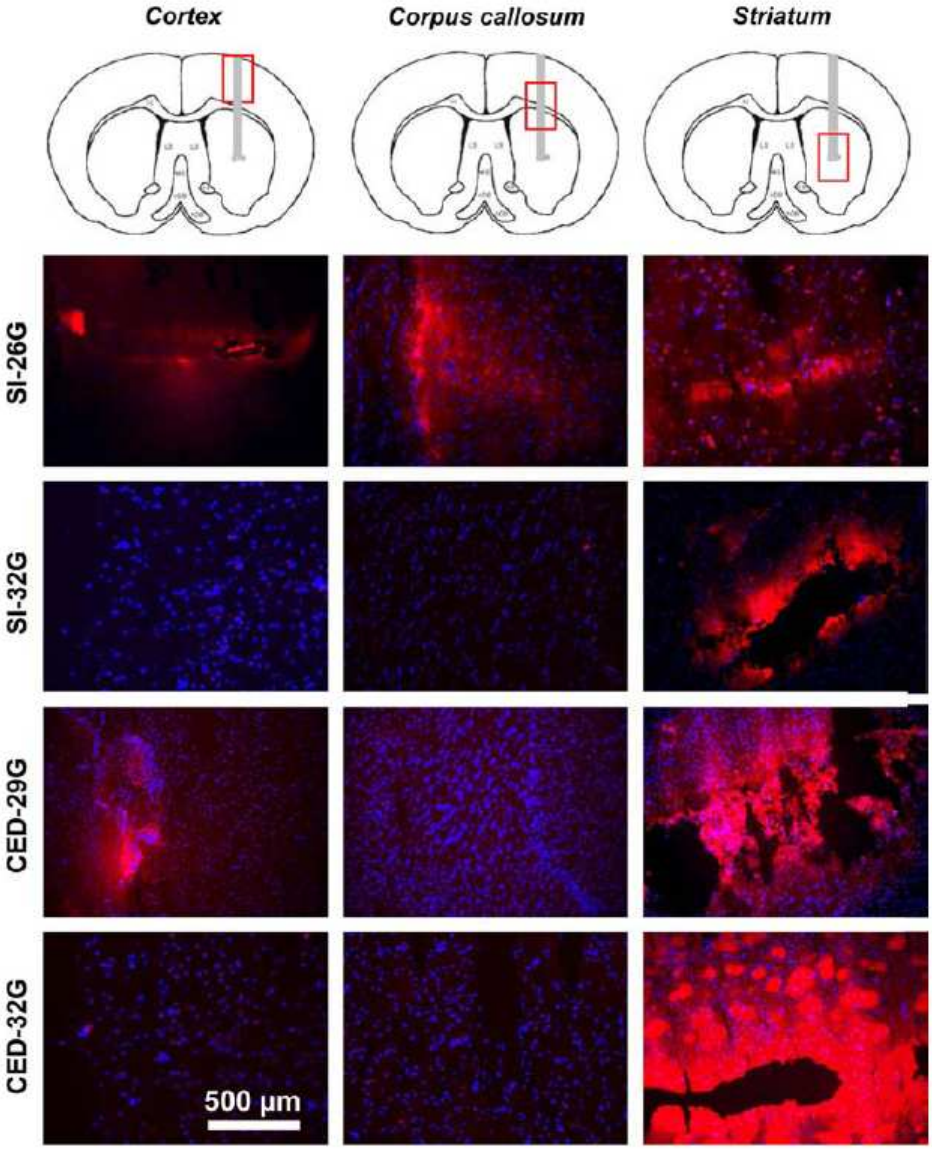

Fig. 3 Representative brain sections seen under fluorescence microscopy to detect the distribution of NR within the rat brain parenchyma at 24 h after intrastriatal stereotaxic implantation of NR-LNC through classical simple injection (SI-26G and SI-32G) or CED (CED-29G and CED-32G). Conventional direct injections of 50 nm-LNC resulted in limited distribution with a high backflow level to the cortical surface (SI-26G). CED-32G significantly enhanced LNC distribution with no back leakage and good homogeneity

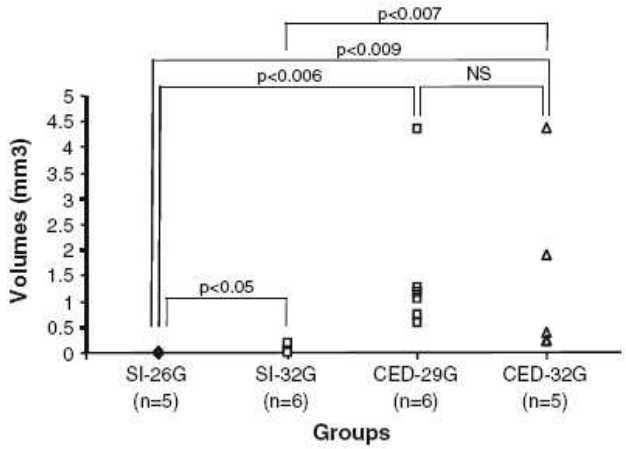

Fig. 4 Calculated NR Vd by measurement of NR-fluorescent surfaces at 24 h after NR-LNC injection. Statistics: *P* indicate the *P*-value obtained from Kruskal–Wallis ANOVA; *NS*, non-significant

from 0.01 ± 0.02 and 0.065 ± 0.07 mm^3 with SI-26G and SI-32G, respectively, to 1.53 ± 1.41 and 1.44 ± 1.79 mm^3 with CED-29G and CED-32G, respectively. This represented a 153-fold improvement with CED-29G ($P < 0.006$) and 144-fold improvement with CED-32G ($P < 0.009$) on the standard stereotaxic injection that we initially used (SI-26G). By eliminating apparent outliers that may create a bias (one from the CED-29G group and two from the CED 32G group, Fig. 4), differences on standard injection were reduced to 115-fold and 36-fold respectively. They were, however, still found significant ($P < 0.009$ and $P < 0.03$ respectively) confirming that the enhanced distribution cannot be attributed only to apparent outliers.

To further study the interest of CED for the intracranial administration of LNC in the case of glioma treatment (tumour-bearing rats), NR distribution from NR-LNC was also followed on a discernible brain tumour. Thus, 6 days after implantation of 10^3 9L cells within the striata of Fischer rats, NR-LNC were injected through CED-32G at the same stereotaxic coordinates. Our data showed that, at 24 h after injection, NR distribution (in red) fully overlapped with the area of tumour progression (cell nucleus/DAPI in blue) (Fig. 5A–D). Interestingly, at higher magnification, NR was found to be associated with a cytoplasmic area avoiding the nucleus of 9L cells in a similar fashion to that of isolated cultured cells (Fig. 5E).

Efficacy of Px-loaded LNC administered intratumourally by CED in the intracranial 9L Fischer rat glioma model

Considering the major significance of radiotherapeutic protocols in the treatment of human glioblastoma [2] and the two main sets of data reported above: firstly, that LNC

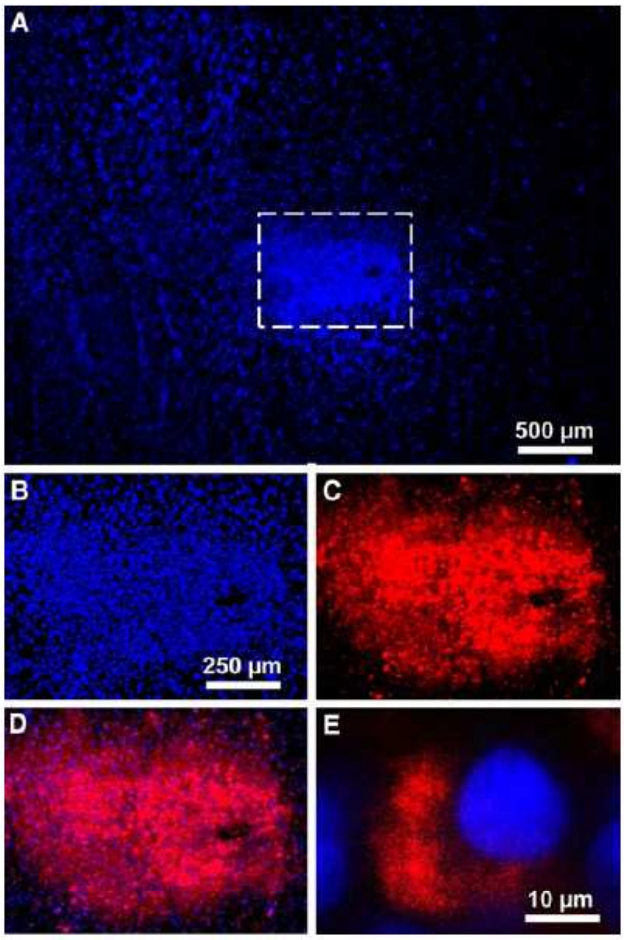

Fig. 5 NR diffusion exemplified on representative section of the rat striatum at 24 h after an intra-tumoural implantation of NR-LNC. **A** DAPI staining illustrating the shape of a 6 day old 9L tumour (initial implantation of 10^3 cells). **B** Higher magnification of the DAPI staining corresponding to the squared area in A. **C** Corresponding NR staining. **D** Merged images. Note the NR fluorescence covering the whole tumour forming area and, at higher magnification, its particular localisation within the cytoplasm of single cells (**E**)

reach the intracellular space of 9L glioma cells and secondly, that CED dramatically improved LNC distribution within the brain parenchyma, we sought to evaluate the impact of an LNC-based radiosensitising chemotherapy administered through CED for the treatment of the 9L intracranial rat glioma model.

For chemotherapy, the hydrophobic compound Px emerged as a good candidate (Fig. 6A). Px is a good radiosensitiser [11] that induces mitotic block at the metaphase/anaphase transition and induces apoptosis [21]. It is poorly soluble in aqueous solution and it weakly crosses the BBB [22, 23]. Its action is concentration and time-dependent [24] and it constitutes the substrate of the MDR-1 efflux pumps which prevent its cytotoxic action [25]. As we previously demonstrated a role for LNC in the augmentation of Px efficacy through improvement of its

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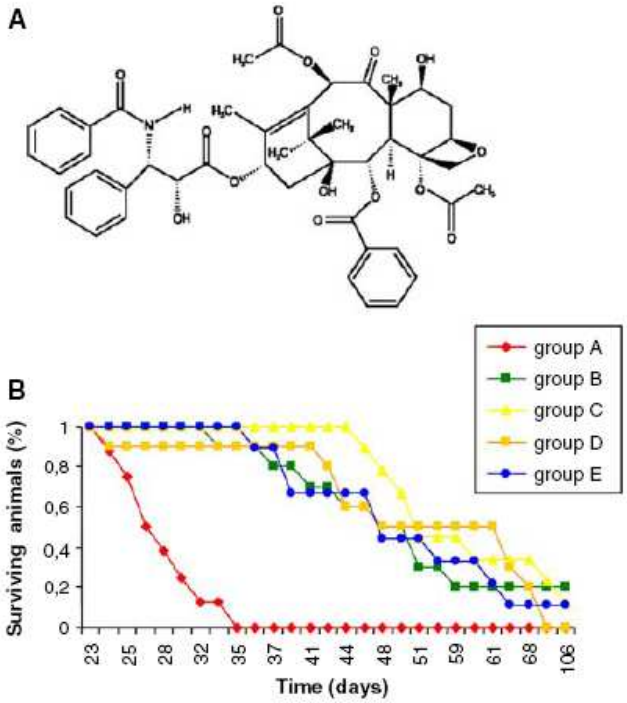

Fig. 6 Efficacy of a nanotechnology-based radiosensitising chemotherapy. **A** Molecular structure of Px. **B** Kaplan–Meier survival curves. Animals with tumours were randomly divided into five groups: **A** untreated, **B** radiotherapy only, **C** CED with Px and radiotherapy, **D** CED with Px-loaded LNC and radiotherapy and **E** CED with unloaded LNC and radiotherapy. Survival curves were established by the Kaplan–Meier method and statistically compared using the Log-rank or Mantel–Cox test (see Table 2)

cellular bioavailability, notably ascribed to the inhibition of the multidrug resistance phenomena [8], Px was selected.

For the different animal groups a systematic clinical follow up-was performed. No clinical toxicity symptoms, including skin necrosis, and no neurological or general changes were observed after treatment, especially after the CED injection of Px-loaded LNC. Radiotherapy started for all the rats on day 8, thus 2 days after CED injection. Not scar disorder delayed the radiotherapy schedule. All rats lost weight just after the end of the radiotherapy schedule but they all recovered their initial weight after a few days. In all cases animals died from their tumour except one rat from group C (free Px) who suffered from neurological deficit and died precociously by day 23 with no evidence of macroscopic tumour in the brain tissue. In all other cases, in which animals were killed after presenting clinical signs of tumour progression, analysis of brain tissue showed no evidence of necrosis within the tumour or within the CNS, notably for the rats receiving the CED of Px-loaded LNC.

Data from the efficacy study are summarised on Kaplan–Meier curves (Fig. 6B). The median survival and long-term survivors (>106 days) of the different groups are indicated in Table 2. Although a significant benefit of radiotherapy

Table 2 Median survival and long-term survivors for each studied group

Groups (n)	Median survival (days)	Long survivors (>106 days)
Group A (8)	26 ± 3.8	0/8
Group B (10)	44 ± 8.2*	2/10
Group C (10)	50 ± 19.3*	0/10
Group D (10)	54 ± 14.8*	1/10
Group E (9)	47 ± 22.8*	1/9

A, untreated; B, radiotherapy only; C, CED with Px and radiotherapy; D, CED with Px-loaded LNC and radiotherapy and E, CED with unloaded LNC and radiotherapy

Statistics: * $P < 0.05$ (Log Rank or Mantel–Cox test)

was observed, only small effects of the different concomitant radiosensitising chemotherapies were superposed. Rats from the control group (group A) presented a median survival of 26 ± 3.8 days. When radiotherapy was given alone (group B), the median survival was 44 ± 8.2 days. When CED with Px-loaded LNC was applied (group D) the median survival reached 54 ± 14.8 days, representing a two-fold increase when compared to the untreated group ($P < 0.01$) that was, however, not statistically different from radiotherapy alone ($P > 0.05$). At last, long-term survivors were observed in three groups only: radiotherapy alone (group B), radiotherapy combined with Px-loaded LNC (group D) and radiotherapy combined with blank LNC (group E).

Discussion

The present study demonstrates for the first time that LNC can be efficiently infused into large volumes of rat brain by CED. It also reveals that LNC perfusion of a whole tumour-forming area inside the CNS is achievable through CED injection whilst preserving the ability of LNC to reach the intracellular space of encountered cells. Although our data attest to the feasibility of the combination of external beam therapy with CED, for the administration of drug-loaded LNC, the use of Px within the nanocarriers emphasises, however, the need for optimisation.

Efficacy of CED technique for LNC injection

While CED proved to be useful for the administration of small molecules or macromolecules in solution within the brain of animals or humans [10, 26], records relating its convenience for the administration of colloidal suspensions are barely emerging. Thus, the CED injection of liposomes was studied for therapeutic or imaging purposes with some success as exemplified by irinotecan [27], pegylated doxorubicin [28], topotecan [29], DNA [30],

adenovirus [31] and gadolinium [17, 32]. However, apart from those liposome focused efforts and a study using a model of polystyrene nanospheres [33], our current investigation represents a primary examination of the diffusion behaviour of nanosystem-based suspensions in solid tissues following CED and at least the first CED brain diffusion study using LNC. The poor distribution of lipophilic drugs could be partly explained by characteristics of the drug itself. In contrast, lipophilic drug distribution became strongly dependent on the characteristics of the carrier when loaded in LNC. Thus, while numerous preclinical studies have demonstrated the efficacy of CED-based local chemotherapy without monitoring the drug distribution [34, 35], our work emphasises the importance of verifying the diffusion of nanoparticle encapsulated drugs within the targeted tissue even when a very promising delivery system is used. At flow rates greater than 1 µl/min, backflow of CED-infused solutions up the outside of the needle shaft has been reported [15]. Backflow reduces control over drug delivery because infused solutions can flow out of the brain or into highly permeable white matter tracts surrounding the infusion site. The separation between tissue and needle that allows backflow can be controlled by adjusting the flow rate and the size of the needle [36]. Although it is conceivable that pressure increases with a reduction of the needle size, our data reveal, however, that the smallest needles (32G) are likely to be less harmful to the tissue and give the best intracranial injection results. This was observed not only for CED-injected LNC suspensions (which fully avoid back leakage) but also with classical stereotaxic injections. Such findings could be ascribed to the nature of the colloidal suspensions that appear to be subjected to pressure constraints distinct from those affecting small molecules or macromolecules in solution [18]. As we learned from liposomes that charge and surface pegylation rather than size affect Vd following CED [29], it is tempting to speculate that the weak charge surface of LNC as well as their embedment by a soft layer form of PEG 660 chains result in a sufficiently low affinity for extracellular matrix components as well as cell surfaces, compatible with a good and homogeneous diffusion. However, LNC diffusion through CED would have to be re-evaluated each time they are modified (e.g. after peptide or antibody grafting) but also each time the incorporated drug would have changed the surface properties of the nanocarrier. In line with this, we previously indicated that all lipophilic components of LNC were mainly hidden by the PEG steric barrier of the PEG-HS at the LNC interface [37]. The fact that the zeta potential for blank-, NR- and Px-LNC remains slightly negative (Table 1) is in favour of similar diffusion behaviours in the brain parenchyma for all those particles. Thus, when encapsulated in LNC, the topographic distribution of Px (not easily detectable per se) must be comparable to the one observed for NR.

Our diffusion work proved that CED allows the injection of large volumes of LNC suspension (60 µl) with minimal tissue change beyond the site of injection and a satisfactory tolerance. In comparison to classical stereotaxic administration, it significantly improves the Vd with no back leakage and good homogeneity. Apparent volumes of distribution are not only multiplied by a factor 6 as expected due to the improvement of the volume injected, but rather by a factor 144. As we previously demonstrated that NR could not penetrate glioma cells on its own [8], the fact that it was incorporated inside most of the cells from the discernible tumour mass supports the notion that LNC preserve their uptake characteristics after CED injection. This also indicated that all tumour tissue was infused with LNC at the beginning of the radiochemotherapeutic treatment investigated. Hence, the administration of LNC through CED within the brain may overcome limitations of traditional treatment for brain tumours caused by the large tumour size and the difficulty of delivering therapeutics into their often dense and heterogeneous tissue [38].

Efficacy and safety of local chemotherapy combined to radiotherapy

The delivery of efficient therapeutic agents to the brain is a major challenge and CED constitutes for this purpose an attractive weapon. Thus, many molecules, usually used systematically in clinical oncology have already been introduced locally by CED injection within the brain: temozolomide [34], topotecan [39], cisplatin [40]. Px has also been combined to CED for investigation in patients but those studies were also largely correlated with a lack of convection, poor tumour response, leakage of the convected drug and treatment-associated complications [41, 42]. As clinical investigations are not performed until there is evidence of efficacy without inordinate toxicity, the poor results of those previous CED trials using Px may have dampened enthusiasm for its use in the upfront setting despite its obvious utility as a radiosensitizing agent. Hence, although the interest of a combination of local chemotherapy with radiotherapy seems undeniable for glioblastoma to enhance effectiveness without increasing total radiation exposure, no study excepted the present preclinical one relates the possible combination of the use of CED for Px injection to external beam therapy.

The advantages of a brain implantation of Px-loaded nanoparticles include bypassing the blood–brain barrier, minimising systemic toxicity, protecting encapsulated Px from surrounding media and obtaining high and sustained concentrations of the drug at the tumour site with only one injection during the whole duration of the radiation

treatment. Our present in vivo work demonstrates that Px-loaded LNC could be safely and effectively used at initial presentation in combination with external beam radiation therapy. However, no benefit of 'radio-chemotherapy' on 'radiotherapy only' is observed. These data contrast with recent in vitro work using Px-loaded PLGA-nanoparticles for radiosensitisation of tumour cells distinct from glioma [43] They are, however, reminiscent of radio-chemotherapeutic clinical experiments on human glioma in which Px was infused intravenously and did not significantly improve survival rates obtained with radiotherapy alone [44–46]. Thus, although the feasibility and the interest of the new technology used in our study are confirmed, there is a need for optimisation. While the biological activity of Px was improved by a factor of 100 on 9L cells in vitro with the LNC formulation [8], the dose of Px administered in the present work remains weak (0.375 mg/kg of bodyweight). This contrasts with doses used with microspheres or implants usually close to 2 mg/kg of bodyweight [47]. Moreover, as LNC have been found to inhibit in the short term the biological activity of efflux pumps interacting with Px [8], possible long-term feed-back-up regulation of those transporters may occur in vivo, as recently proven when chemotherapy was combined with radiotherapy [48]. Finally, it should be stressed that clinical protocols have to be strongly inspired from the current advances made in the comprehension of what brain cancer really is. The recent hypothesis that tumourigenicity is conferred by a rare subpopulation of cancer cells, the 'brain tumour stem cells' [49] and the fact that hypoxia inhibits Px-induced apoptosis [50] should all be taken into account for more specific and reliable strategies. To address these concerns, one has to consider that LNC are not inert entities but real interactive 'nano-cargo'. Thus, for CED optimisation, the possibility of grafting molecular recognition moieties (e.g. antibodies, peptides, aptamers) onto their surface to reach specific cells or biomolecular components [51] combined to their biocompatible nature (no use of organic solvents) is of considerable interest.

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✓ **Abstract n° 4**

- Combined effects of radiotherapy and paclitaxel-loaded lipid nanocapsules infused by convection-enhanced delivery in the 9L intracranial rat glioma model.
Vinchon-Petit. S, Jarnet D, Paillard A, Benoit JP, Garcion E, Menei P.
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Combined effects of radiotherapy and paclitaxel-loaded lipid nanocapsules infused by convection-enhanced delivery in the 9L intracranial rat glioma model.

S. Vinchon-Petit[a], D.Jarnet[c], A. Paillard[a], JP. Benoit[a], E. Garcion[a], P. Menei[a,b]

[a] *Inserm U646, 10 rue André Boquel, Université d'Angers, 49100 - Angers, France*

[b] *Département de Neurochirurgie, CHU, 4 rue Larrey, 49033 - Angers, France*

[c] *Centre Régional de Lutte Contre le Cancer, Centre Paul Papin, 2 rue Moll, 49933 - Angers, France.*

Malignant gliomas are devastating tumours from central nervous system associated with poor prognosis. Although usual treatment includes surgery when possible followed by external beam therapy, new biomedical strategies capable to address local invasion are highly required. Convection-enhanced delivery (CED) is a novel regional method that allows distribution of substances throughout the interstitium via positive-pressure infusion. Thus, the objective of the present study was to investigate its potential interest for the intratumoural administration of a nanotechnology-based radiosensitizing chemotherapy in the rat. Firstly, pursuing on newly developed biocompatible lipid nanocapsules (LNC) that can improve efficacy of encapsulated drug together with revert multidrug resistance (1, 2), we have studied their intracerebral diffusion through CED when fluorescently-labeled with the hydrophobic compound Nile red. At 24 hours after injection, microscope slide analysis demonstrated that CED allows the injection of important volumes of LNC suspension (60 µL) with a satisfactory tolerance. In comparison to classical stereotactic administration, it improves significantly the distribution volume (144-fold) with no back leakage and good homogeneity. Secondly, as paclitaxel may be an interesting radiosensitizer acting on G2-M phase of cell division, we evaluated the potential radiosensitizing effects of paclitaxel-loaded LNC administered through CED in the 9L intracranial rat glioblastoma model. Animals with tumours were randomly divided in five groups: untreated, radiotherapy only, CED with paclitaxel and radiotherapy, CED with paclitaxel-loaded LNC and radiotherapy and CED with unloaded LNC and radiotherapy. Survival curves were established by the Kaplan-Meyer method and statistically compared using the Log Rank test. We have established an animal model of encephalic irradiation close from the clinic. Although the "CED with paclitaxel-loaded LNC" group had the best median of survival of 54 days (twice more than the untreated group), the addition of a free or encapsulated local chemotherapy did not improve significantly the survival. While CED constitutes an attractive weapon against glioblastoma, the LNC concept remains perfectible.

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La technique de la CED et le schéma de radiothérapie encéphalique du rat ont été utilisés dans d'autres études menées au laboratoire notamment dans l'article qui suit auquel j'ai collaboré. Il est une autre illustration de tentative d'amélioration de la radiosensibilisation des tumeurs gliales de haut grade par agent thérapeutique encapsulé et implanté localement.

✓ Article n°6

- Local delivery of ferrociphenol lipid nanocapsules followed by external radiotherapy as a synergistic treatment against intracranial 9L glioma xenograft.
Allard E, Jarnet D, Vessières A, Vinchon-Petit S, Jaouen G, Benoit JP, Passirani C. Pharm Res. 2010 Jan; 27(1):56-64.

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Research Paper

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

Emilie Allard,[1] Delphine Jarnet,[2] Anne Vessières,[3] Sandrine Vinchon-Petit,[1,2] Gérard Jaouen,[3] Jean-Pierre Benoit,[1] and Catherine Passirani[1,4]

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Purpose. The goal of the present study was to evaluate the efficacy of a new organometallic drug, ferrociphenol (Fc-diOH), in combination with external radiotherapy in intracerebral 9L glioma model. We tested the hypothesis that the combination of external radiotherapy with Fc-diOH could potentiate the action of this drug.

Methods. 9L cells were treated with Fc-diOH-LNCs (from 0.01 to 1 µmol/L) and irradiated with external radiotherapy (from 2 to 40 Gy). *In vivo* assessment was evaluated by the inoculation of 9L cells in Fisher rats. Chemotherapy with Fc-diOH-LNCs (0.36 mg/rat) was administered by means of convection-enhanced delivery (CED), and the treatment was followed by three irradiations of 6 Gy doses (total dose=18 Gy).

Results. *In vitro* evaluations evidenced that a combined treatment with Fc-diOH-LNCs and irradiations showed synergistic antitumor activity on 9L cells. Combining cerebral irradiation with CED of Fc-diOH-LNCs led to a significantly longer survival and the existence of long-term survivors compared to Fc-diOH-LNCs-treated animals ($p<0.0001$) and to the group treated with blank LNCs+radiotherapy ($p=0.0079$).

Conclusion. The synergistic effect between ferrociphenol-loaded LNCs and radiotherapy was due to a closely oxidative relationship. Upon these considerations, Fc-diOH-LNCs appear to be an efficient radiosensitive anticancer drug delivery system.

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

INTRODUCTION

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

[1] Inserm U646, Pôle pharmaceutique, CHU d'Angers, Université d'Angers, 49100 Angers, France.
[2] Centre Régional de Lutte Contre le Cancer, Centre Paul Papin, 49933 Angers, France.
[3] UMR CNRS 7223, Ecole Nationale Supérieure de Chimie de Paris, 75231 Paris cedex 05, France.
[4] To whom correspondence should be addressed. (e-mail: catherine.passirani@univ-angers.fr)

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

MATERIALS AND METHODS

Materials

Ferrocenyl diphenol compound (2-ferrocenyl-1,1-bis(4-hydroxyphenyl)-but-1-ene) named Fe-diOH was prepared by a McMurry coupling reaction (21). The lipophilic Labrafac® CC (caprylic-capric acid triglycerides) was kindly provided by Gattefosse S.A. (Saint-Priest, France). Lipoïd® S75-3 (soybean lecithin at 69% of phosphatidylcholine) and Solutol® HS15 (a mixture of free polyethylene glycol 660 and polyethylene glycol 660 hydroxystearate) were a gift from Lipoïd Gmbh (Ludwigshafen, Germany) and BASF (Ludwigshafen, Germany), respectively.

Preparation of Fe-diOH-LNCs

Lipid nanocapsules were prepared according to our previously described procedure (16). Briefly, Solutol® HS15 (17% w/w), Lipoid® (1.5% w/w), Labrafac® (20% w/w), NaCl (1.75% w/w) and water (59.75% w/w) were mixed and heated under magnetic stirring up to 85°C. Three cycles of progressive heating and cooling between 85°C and 60°C were then carried out and followed by a dilution with 2°C deionised water added to the mixture at 70–75°C. To formulate Fe-diOH-LNCs, a first step consisted in dissolving the anticancer drug in triglycerides (Labrafac®) using ultrasound during 15 h. Two parameters were varied: the amount of Fe-diOH in triglycerides (1.7% and 4% (w/w)) and the volume of cold water for LNC dilution (70% and 28.5% v/v) to obtain drug loadings of 1 mg/g (0.84% w/w dry weight) and 6.5 mg/g (2% w/w dry weight), respectively. For *in vivo* applications combined with radiotherapy, sucrose (20% w/w) was dissolved in the aqueous phase of the LNC suspension after formulation.

LNC Physicochemical Properties

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

Tumor Cell Line

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

Irradiation

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

Cell Protocol

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

Protocol RT+CT *versus* CT+RT

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

Combined Effect Protocol

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

Multi-Irradiation Protocol

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

MTT Survival Test

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

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

Animals and Intracranial Xenograft Technique

Syngeneic Fischer F344 female rats weighing 160–180 g were obtained from Charles River Laboratories France (L'Arbresle, France). All experiments were performed on 10 to 11-week old female Fisher rats. The animals were anesthetized with an intraperitoneal injection of 0.75–1.5 ml/kg of a solution containing 2/3 ketamine (100 mg/ml) (Clorketam®, Vétoquinol, Lure, France) and 1/3 xylazine (20 mg/ml) (Rompun®, Bayer, Puteaux, France). Animal care was carried out in strict accordance with French Ministry of Agriculture regulations. A 9L tumor monolayer was detached with trypsin-ethylenediamine tetraacetic acid, washed twice with EMEM (Eagle's Minimal Essential Medium) without FCS or antibiotics, counted and resuspended to the final concentration desired. For intracranial implantation, 10 microliters of 1,000 9L cell suspension were injected into the rat striatum at a flow rate of 2 μl per minute using a 10 μl syringe (Hamilton® glass syringe 700 series RN) with a 32G needle (Hamilton®). For that purpose, rats were immobilized in a stereotaxic head frame (Lab Standard Stereotaxic; Stoelting, Chicago, IL). A sagittal incision was made through the skin, and a burr hole was drilled into the skull with a twist drill. The cannula coordinates were 1 mm posterior from the bregma, 3 mm lateral from the sagittal suture and 5 mm below the dura (with the incisor bar set at 0 mm). The needle was left in place for 5 additional minutes to avoid expulsion of the suspension from the brain during removal of the syringe, which was withdrawn very slowly (0.5 mm per minute).

Convection-Enhanced Delivery

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

Statistical Analysis

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

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

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

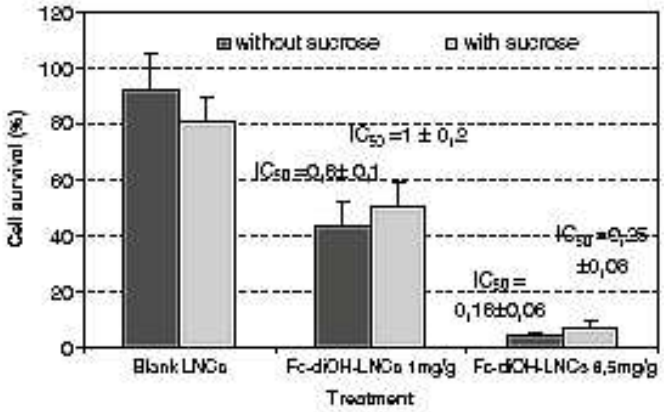

Fig. 1. Cell survival test after treatment with Fe-diOH-loaded and blank LNCs with or without sucrose in external phase. Formulations were diluted in culture medium with the same dilution factor (1/2350). Final concentrations of Fe-diOH were equivalent to 0, 1 and 6.5μmol/L for Blank LNCs, Fe-diOH-LNCs 1 mg/g and Fe-diOH-LNCs 6.5 mg/g, respectively. IC50 values were about 0.8 to 1μM for Fe-diOH-LNCs 1 mg/g and about 0.18 to 0.25μM for Fe-diOH-LNCs 6.5 mg/g without and with sucrose, respectively.

cant). The Kaplan-Meier method was used to plot animal survival. Statistical significance was calculated using the log-rank test (Mantel-Cox Test). StatView software version 5.0 (SAS Institute Inc.) was used for that purpose, and tests were considered as significant with p values <0.05. The different treatment groups were compared in terms of median survival time (days), increase in survival time (IST_{median} %), and long-term survivors (%).

RESULTS

LNC Formulation and Cytotoxicity

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

Combined Effects of Chemotherapy and Radiotherapy

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

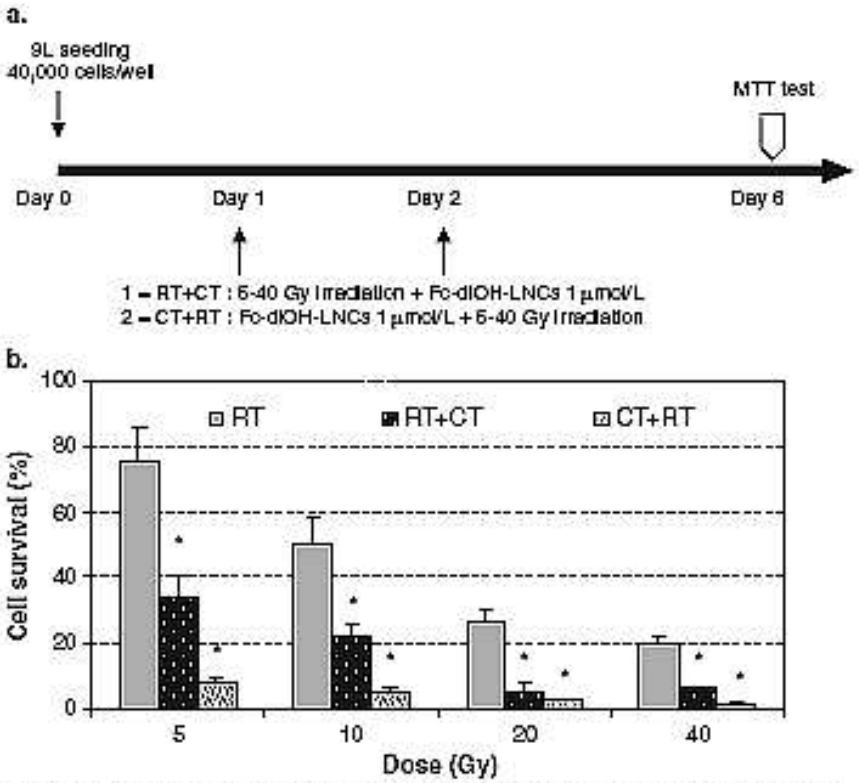

Fig. 2. Cell survival percentage of 9L cells according to two distinct protocols: radiotherapy+chemotherapy (RT+CT: protocol 1) versus chemotherapy+radiotherapy (CT+RT: protocol 2). Chemotherapy was a treatment with Fe-diOH-LNCs 1 mg/g at 1μmol/L, and Radiotherapy was a single irradiation at 5, 10, 20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

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

Synergistic Effects

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

Multi-Irradiation

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

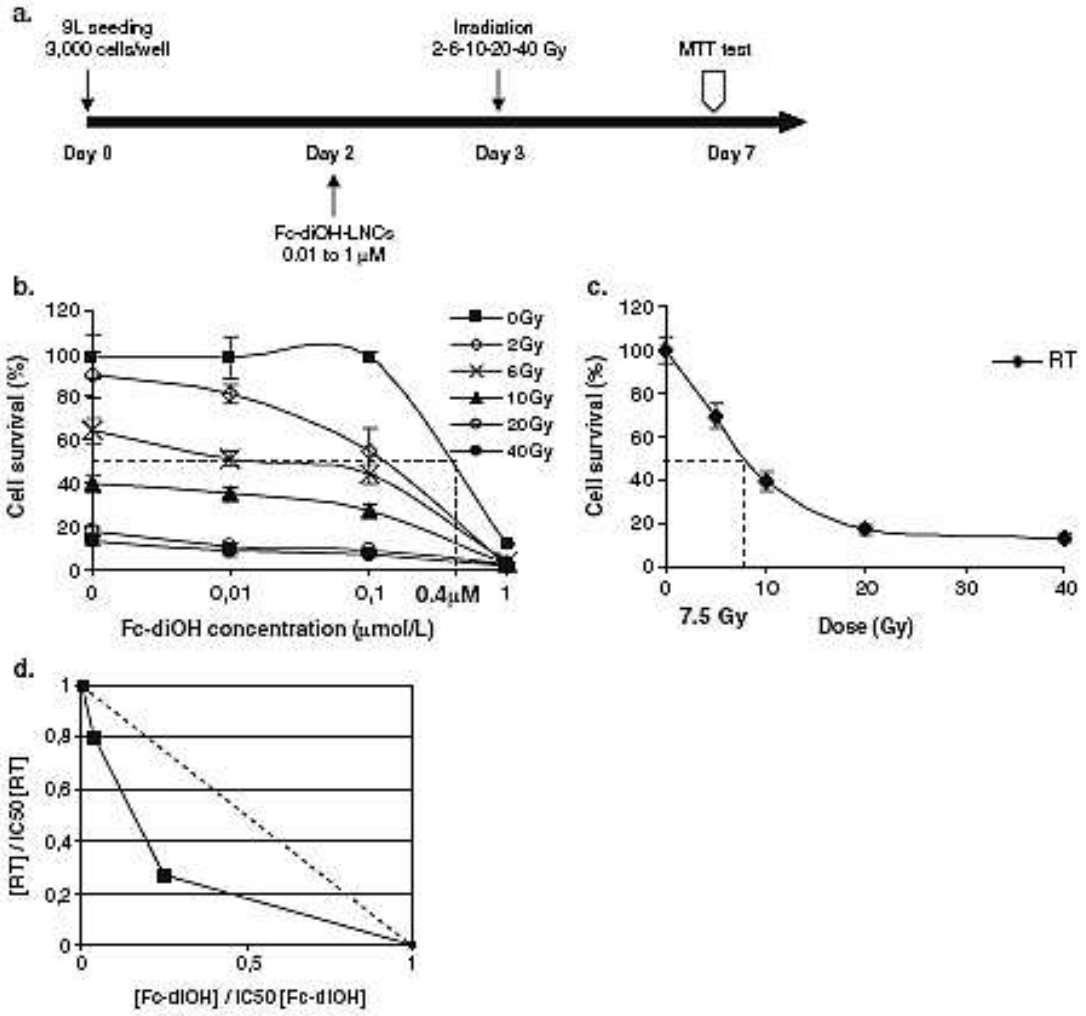

Fig. 3. Synergistic effect of cell death by Fe-diOH-LNCs and radiotherapy in 9L glioma cells. Cells were treated according to the protocol detailed in (a). Cell survival percentage of 9L cells treated with CT (from 0.01 to 1 µM)+ RT (from 2 to 40 Gy) were expressed in (b). IC_{50} for CT alone (RT=0Gy)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

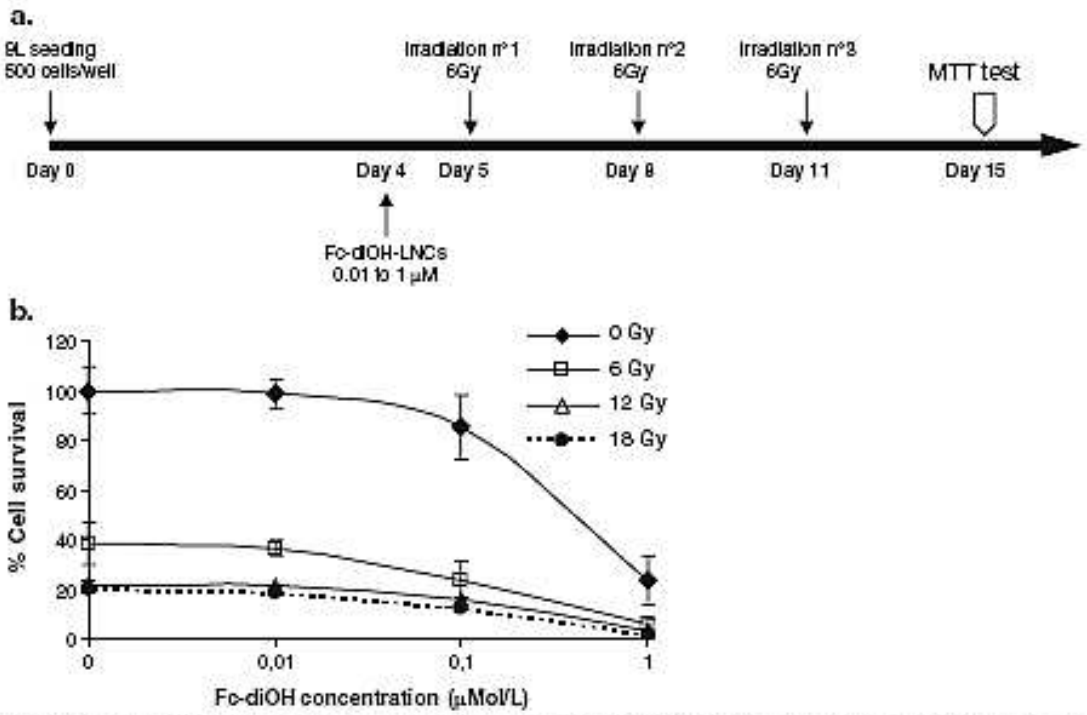

Fig. 4. 9L cell viability after a chemotherapy treatment with Fe-diOH-LNCs followed by a multi-irradiation scheme of 3x6 Gy. Multi-irradiation protocol is detailed in (a). (b) represents the cell survival percentage after treatment with Fe-diOH-loaded LNCs at concentrations between 0.01 and 1µM and followed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

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

Survival Study

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

DISCUSSION

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

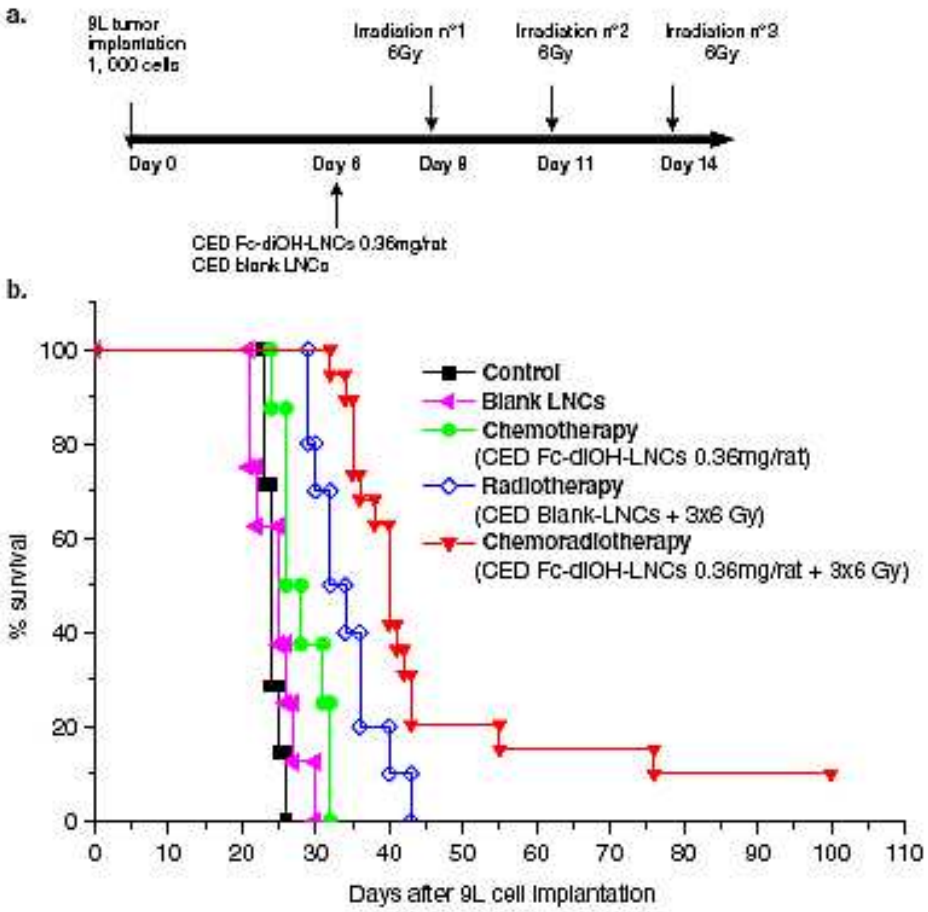

Fig. 5. Representation of the chemoradiotherapy protocol applied on 9L glioma-bearing rats (a). Kaplan-Meier survival curves for 9L glioma-bearing rats after CED of Fc-diOH-LNCs and external radiotherapy 3x6 Gy (b). Survival times in days after tumor implantation have been plotted for untreated animals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

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

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

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

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

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

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

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

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

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

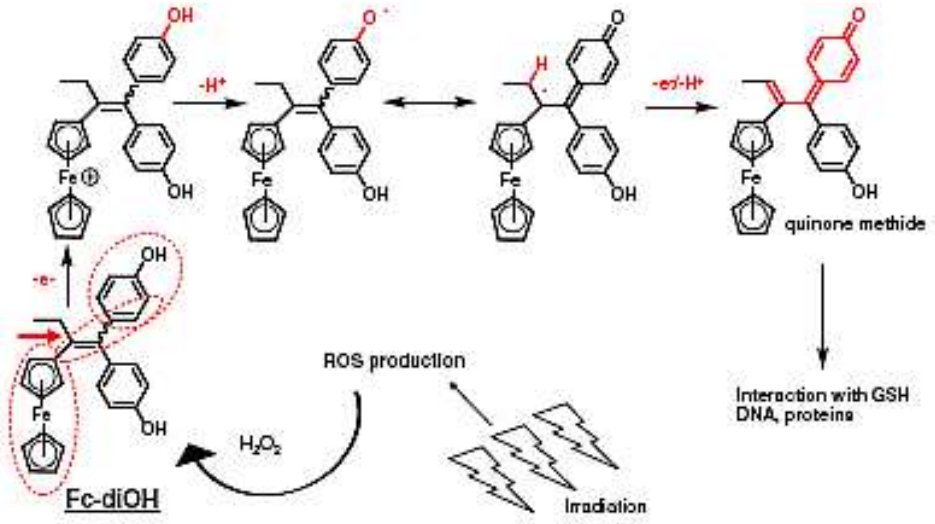

Fig. 6. Proposed mechanism for Fc-diOH cytotoxicity based on electron-transfer studies. Irradiation by the generation of reactive oxygen species (ROS) can form hydrogen peroxide (H_2O_2). H_2O_2, which is a strong oxidizing molecule, can boost the oxidation of ferrocene in ferrocenium, which, after rearrangement,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

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

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

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b. Discussion

Notre étude est la première basée, d'une part, sur le pouvoir radiosensibilisant du paclitaxel injecté en intracérébral et d'autre part, sur la vectorisation par nanoparticules de cet agent, in vivo, par CED dans la prise en charge du glioblastome. Le groupe ayant bénéficié d'une CED de paclitaxel présente le temps moyen de survie le plus élevé devant le groupe «LNC paclitaxel + RTE». L'introduction de cette chimiothérapie locale sous forme libre permet une augmentation de survie de 92% par rapport à l'absence de traitement mais surtout on observe une augmentation de 12% de la survie par rapport au groupe «radiothérapie seule». Le paclitaxel en solution confirme donc son pouvoir radiosensibilisant et sa bonne tolérance dans la prise en charge du glioblastome. Il pourrait être intéressant de comparer cette efficacité avec d'autres drogues elles aussi liposubles dont l'action est limitée par l'utilisation de la voie IV ou même avec des agents qui, même s'ils pénètrent la BHE, possèdent une courbe de réponse thérapeutique fortement dose-dépendante tels que le témozolomide par exemple. L'utilisation de témozolomide administré par CED a fait l'objet d'une étude par Heimberger sur un modèle de tumeurs xénogreffées de type D54 (83). Ce traitement permettait l'amélioration de la survie ($p<0,001$) avec rémissions clinique et histologique complète. Cependant, les réserves émises dans cet article concernaient le défaut de solubilité de la drogue employée. L'encapsulation pourrait combler ce déficit. De plus, compte tenu de l'effet additif du témozolomide associé à la radiothérapie (92), il pourrait constituer un meilleur candidat pour notre étude.

En effet**,** bien que le groupe "CED avec LNC de paclitaxel" présente la meilleure médiane de survie avec 54 jours (deux fois plus que celle du groupe non traité), le fait d'injecter localement, en intratumoral, du paclitaxel encapsulé n'améliore pas significativement la survie. Alors que la CED constitue une arme attractive dans la lutte contre le glioblastome, le concept des LNC demeure perfectible.

Un des intérêts du procédé de formulation des LNC est qu'il n'implique pas l'utilisation d'un solvant organique. Cependant, la faible solubilité au sein du Labrafac® CC des principes

actifs, pourtant hydrophobes, tels que le paclitaxel, n'a pas permis de s'affranchir de son utilisation. Le choix des solvants utilisés a été fait en fonction, d'une part, des caractéristiques de solubilité des principes actifs considérés et, d'autre part, des caractéristiques toxicologiques des solvants considérés. De plus, la possibilité d'élimination des solvants par évaporation lors de la fabrication des LNC, notamment lors des cycles de chauffage, a été prise en compte. L'éthanol a permis ainsi de formuler des nanocapsules faiblement chargées en paclitaxel (0.1% ; m/m) et pour des charges supérieures (1-2% ; m/m), l'utilisation de cholestérol et d'un co-solvant, le dichlorométhane, a été nécessaire. Pour les nanocapsules chargées, les résultats de leur caractérisation montrent que l'emploi de solvants organiques n'a pas altéré le processus d'inversion de phases. Il semble également que la tolérance cérébrale des LNC ne soit pas remise en question. Les LNC de paclitaxel n'entraînent aucun déficit général ou neurologique comparées aux LNC blanches.

Les LNC blanches sont responsables d'une augmentation de survie de 81%, probablement par leur capacité à interagir avec les pompes à efflux et à priver les cellules tumorales d'un de leur mécanisme de résistance. Ces résultats confirment ceux obtenus par les études *in vitro*. Mais même si les LNC permettent la libération lente et prolongée d'agents cytotoxiques *in vitro*, il nous manque des données *in vivo* sur leur fonctionnement. Nous avons observé que les LNC sont internalisées au sein du compartiment intra-cellulaire des cellules tumorales (Photo 12). Elles se retrouvent donc à l'endroit où le paclitaxel, une fois libéré, pourra atteindre facilement sa cible que sont les microtubules du fuseau mitotique. Ainsi, ce n'est pas le lieu qui ne convient pas mais peut-être davantage la cinétique de libération du paclitaxel.

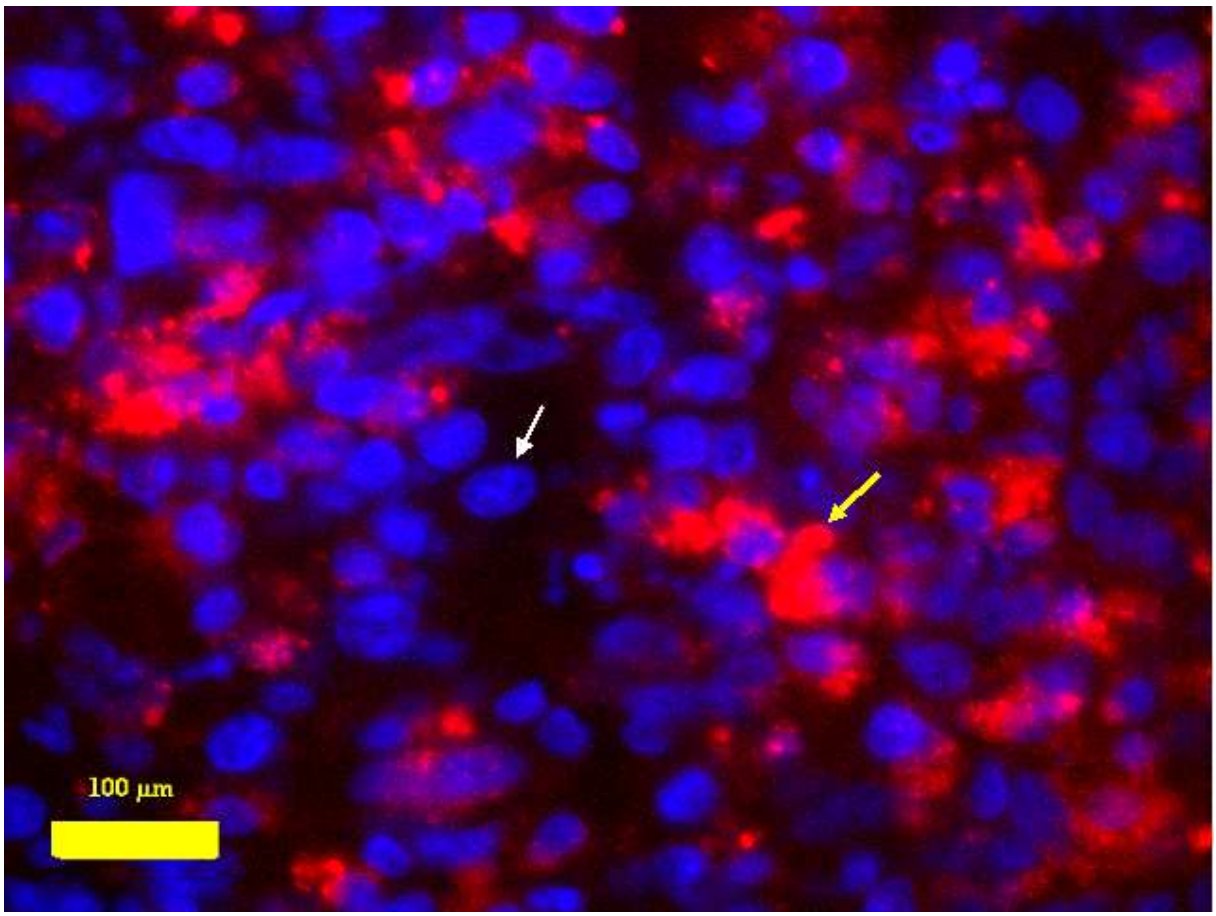

Photo 12: LNC fluorescentes chargées en Nile Red localisées au sein des cellules striatales des rats injectés par CED.

Nous avons démontré précédemment que 10% de la dose de paclitaxel contenue dans les LNC est relarguée sur 21 jours. Ainsi, malgré la quantité importante de LNC injectée sur le site tumoral grâce à la CED, la concentration de paclitaxel obtenue ne semble pas suffisante pour être efficace. Il est difficile de connaître la posologie exacte nécessaire en injection locale puisque cette voie d'administration n'est pas utilisée en routine. Les seules données dont nous disposons sont celles concernant la voie intra-veineuse.

La difficulté technologique rencontrée pour isoler les LNC nous a empêchés de calculer les véritables taux d'encapsulation des différents principes actifs étudiés. Cependant, le calcul du rendement de récupération pour le paclitaxel et le caractère fortement hydrophobe de cet agent

laissent supposer qu'il est encapsulé en totalité au sein des LNC. La capacité de charge en paclitaxel des LNC par rapport à d'autres types de vecteurs est intéressante. Il a été possible de formuler des LNC chargées jusqu'à 1 et 2 % en masse (i.e. 0.7 à 1.4 % en moles) par rapport aux constituants lipidiques soit une quantité totale de 10 à 20 mg de paclitaxel par préparation (i.e. 11.7 à 23.4 µmol) avec une concentration finale de 2 à 4 mg/ml de paclitaxel dans la suspension de LNC. Ces résultats sont à comparer avec ceux obtenus avec d'autres systèmes nanoparticulaires chargés en paclitaxel tels que des nanocapsules et des liposomes de concentration 0.6 à 1 mg/ml (93). Une façon de palier le manque d'efficacité de nos LNC serait d'augmenter davantage la concentration de paclitaxel encapsulée. Malheureusement, il est difficile d'améliorer ce paramètre sans interférer avec la stabilité des LNC.

Certaines formulations de liposomes ont permis d'obtenir des vecteurs de charges molaires de 3 % en paclitaxel supérieures à celles obtenues avec les LNC. Certains auteurs ont cependant réussi à incorporer dans des nanosphères des proportions massiques de paclitaxel largement supérieures à celles obtenue avec les LNC (94). Cependant, ces préparations semblent posséder soit une toxicité potentielle importante, soit être responsable lors des études de libération *in vitro* de phénomène de burst non négligeable (94). A l'inverse, une phase de libération initiale plus lente observée avec les charges les plus fortes en principe actif (LNC formulées avec une charge en paclitaxel de 1 % comparée à celle formulées avec une charge de 0.1%) peut être observée et serait due à un phénomène déjà observé et modélisé (95). Ces auteurs proposent l'hypothèse de cristallisation du principe actif au sein du vecteur nanoparticulaire lorsqu'il est présent en quantité importante. Parallèlement, d'autres systèmes nanoparticulaires ayant des propriétés proches des LNC, notamment en ce qui concerne l'inhibition de la MDR, ont été développés mais n'ont jamais été testés en CED ni en association avec la radiothérapie: micelles de copolymères blocs synthétiques (96) et nanoparticules de poly (alkyl cyanoacrylate) (97).

3. CONCLUSION FINALE

Le concept de thérapie locale est séduisant dans la prise en charge des tumeurs gliales de haut grade. Il permet d'outrepasser la BHE et d'obtenir un index thérapeutique élevé au niveau du site tumoral initial, là où se développeront les récidives.

Le gliadel® est le premier implant polymérique à détenir l'AMM dans le traitement des glioblastomes nouvellement diagnostiqués ou récidivants. Il libère de la carmustine de façon progressive et contrôlée.

Cependant, son influence sur la survie reste modeste et sa tolérance n'est pas parfaite. Il peut être responsable de nécrose symptomatique. De plus, sa conformation empêche la diffusion intratumorale du principe actif puisqu'il n'est qu'apposé dans la cavité opératoire.

D'autres vecteurs administrés par injection intracérébrale ont été développés et testés chez l'animal. D'autres agents cytotoxiques ont été évalués.

Déjà connues dans la prise en charge des tumeurs hépatiques primitives ou secondaires, les microsphères de doxorubicine (1 mg/ml) et d'irinotécan (100 mg/ml) produites par Biocompatibles sont bien tolérées une fois injectées dans le parenchyme cérébral. Même si leur effet radiosensibilisant n'a pas été à la hauteur de ce que nous espérions, nous avons noté que les microsphères d'irinotécan amélioraient très significativement la survie des rats traités par rapport aux rats contrôles. Ces résultats ont permis à Biocompatibles de prétendre à la mise en place d'une étude clinique de phase I. La constitution du dossier destiné à l'EMA est en cours.

Nos LNC sont produites par inversion de phase et peuvent être chargées avec différents produits. Nous avons démontré une tendance à l'augmentation de la survie des rats en faveur des LNC de paclitaxel en association avec la radiothérapie (+10 jours par rapport au groupe irradié seul). Bien que les résultats soient non significatifs, le concept des LNC est séduisant notamment lorsqu'il est couplé à la CED qui permet l'injection intracérébrale de volumes significativement plus importants avec une tolérance satisfaisante. Si l'on souhaite exploiter au maximum l'ensemble de ses ressources, il reste maintenant à optimiser les connaissances sur ce vecteur afin

avec notamment possibilité d'adressage subcellulaire et amélioration de la cinétique de libération du principe actif.

Quoiqu'il en soit, compte-tenu des résultats obtenus en clinique, l'amélioration de nos outils de thérapie locale ne pourra se faire sans la participation des thérapies ciblées dont le chef de file en neuro-oncologie est le bévacizumab. Il pourrait être intéressant d'étudier l'association de microsphères d'irinotécan injectées localement à du bévacizumab introduit par voie systémique. Ce schéma permettrait-il l'amélioration des résultats obtenus actuellement avec la combinaison de ces deux molécules par voie générale? Puisque le bénéfice de ce traitement semble lié en grande partie au bévacizumab, l'emploi de l'irinotécan sous forme encapsulée pourrait peut-être renforcer sa propre action.

Tout comme des efforts sont faits pour affiner nos connaissances sur la biologie moléculaire des tumeurs gliales de haut, des efforts sont essentiels dans l'amélioration de nos outils de traitement. L'amélioration du pronostic des gliomes passe aussi par la multiplication des armes thérapeutiques quelles soient locales ou systémiques.

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5. LEGENDES

FIGURES

Figure 1: Représentation des propriétés infiltrantes des tumeurs gliales de haut grade (en situation pré-opératoire).

Figure 2: Brachythérapie adaptée aux tumeurs cérébrales sous forme d'implants d'iode radioactif ou de cathéter intracrânien délivrant de l'iode radioactive sous forme liquide (Gliasite®).

Figure 3: Représentation d'un montage de Convection-Enhanced Delivery en pratique préclinique. Adaptation sur le cadre de stéréotaxie du matériel d'injection relié à la pompe.

Figure 4: Formule chimique de l'acide poly (1,3-bis (carboxyphénoxy) propane-co-sabacique (Polifeprosan®), polymère utilisé dans la formulation du Gliadel®.

Figures 5A et B: Procédé de fabrication des nanocapsules lipidiques (LNC).

Figure 6: Représentation schématique des différents systèmes de thérapie locale et leur diffusion. a: Implants monolithiques de Gliadel ®; b: Injection de microsphères de 5-Fu; c: Injection de substances en solution par CED.

Figure 7: Procédé de chargement des microsphères en doxorubicine

Figure 8: Exemples de microsphères chargées en doxorubicine disponibles sur le marché variant en taille et en concentration.

Figure 9: Illutrations des propriétés des LNC, notamment le phénomène anti-MDR.

PHOTOS

Photo 1: Injection locale directe intra-parenchymateuse cérébrale à la seringue d'un soluté en per opératoire.

Photos 2A et B: Illustration d'un montage de Convection-Enhanced Delivery en pratique clinique. A: Implantation au sein du parenchyme cérébral de cathéters. B: Montage reliant les cathéters au système de pompe à l'aide de tubulures étanches.

Photo 3: Premières applications cliniques des éponges de gélatine imbibées d'agents cytotoxiques.

Photo 4: Implant de Gliadel®.

Photo 5: Microsphères de 5-FU en suspension.

Photo 6: Exemple de chargement des microsphères (300-500 µm) en doxorubicine à 1 minute (a), 10 minutes (b) et 20 minutes (c).

Photo 7: Seringue Hamilton classiquement utilisée pour les injections intracrâniennes chez le rat.

Photo 8: Différences de concentrations en microsphères selon la méthode de prélèvement utilisée. Gauche: Deux gouttes de 10µl de solution de microsphères provenant d'une pipette avec cône jaune; Droite: Deux gouttes de 10 µl de solution de microsphères provenant d'une seringue Hamilton (21G).

Photo 9: Montage de CED chez le rat pour les injections intracrâniennes.

Photo 10: Injections intracrâniennes des microsphères blanches de Biocompatibles avec une seringue à tuberculine Kendall Monoject de 1 ml montée d'une aiguille BD Hypodermique 0,6x 25mm de 23G.

Photo 11: Nécrose cérébrale observée chez nos rats traités par les CM-BC1 à la concentration de 10 mg/ml.

Photo 12: LNC fluorescentes chargées en Nile Red localisées au sein des cellules striatales des rats injectés par CED.

SCHEMAS

Schéma 1: Cinétique de libération du 5-FU par les microsphères.

Schéma 2: Schéma thérapeutique (bras RTE+TMZ) évalué dans l'essai randomisé de phase III mené par l'EORTC et le NCIC (Schéma de STUPP) (7).

RESUME

Une des difficultés rencontrées lors de l'administration d'agents thérapeutiques au sein du parenchyme cérébral peut être résolue par l'utilisation de nouvelles formes galéniques implantables. Nous montrons dans ce travail la biocompatibilité dans le cerveau de rat des microsphères synthétisées par Biocompatibles facilement injectées par stéréotaxie et chargées en doxorubicine (1mg/ml) (CM-BC1) ou en irinotécan (100mg/ml) (CM-BC2). Nous avons ensuite rapporté l'efficacité des CM-BC2 sur le gliome 9L du rat avec une amélioration significative de la survie par rapport aux groupes non traité ou traité par microsphères blanches. La combinaison des traitements n'améliore pas significativement la survie par rapport à la radiothérapie seule. La récente expansion des nanotechnologies associée aux progrès des méthodes mini-invasives d'administration intracérébrale de médicaments offre l'opportunité d'améliorer ces résultats. Nous avons étudié, toujours sur lemodèle 9L, l'effet radiosensibilisant du paclitaxel, injecté en intratumoral par Convection-Enhanced Delivery (CED) et vectorisé par des nanocapsules lipidiques (LNC) développées par le laboratoire. Une tendance à l'augmentation se profile en faveur des LNC de paclitaxel qui permettent l'obtention de la meilleure médiane de survie (+10 jours par rapport au groupe irradié seul). Bien que les résultats soient non significatifs, l'association d'une chimiothérapie locale à la radiothérapie est intéressante. Il reste maintenant à optimiser les connaissances sur ce vecteur avec notamment possibilité d'adressage subcellulaire et amélioration de la cinétique de libération du principe actif.

Mots Clés :
Irinotécan, Doxorubicine, Paclitaxel, microsphères, nanocapsules, gliome, radiosensibilisation.

Difficulties in drug administration in the CNS can be resolved by the use of new polymeric devices. This work demonstrates that beads loaded with doxorubicin (1mg/ml) (CM-BC1) or with irinotecan (100mg/ml) (CM-BC2) and developed by Biocompatible are safe when implanted in rat brain by stereotaxy. CM-BC2 are efficient on 9L rat glioma with a significant improvement of survival compared to the untreated and unloaded beads groups. But the combination of treatments isn't as good as attempted compared to the radiation therapy. The development of nanotechnology-based chemotherapy may improve these results. Pursuing on newly developed biocompatible lipid nanocapsules (LNC) that can improve efficacy of encapsulated drug together with revert multidrug resistance, we evaluated the potential radiosensitizing effects of paclitaxel-loaded LNC administered through CED in the 9L intracranial rat glioblastoma model. Although the "CED with paclitaxel-loaded LNC" group had the best median of survival of 54 days (twice more than the untreated group), the addition of a free or encapsulated local chemotherapy did not improve significantly the survival. While CED constitutes an attractive weapon against glioblastoma, the LNC concept remains perfectible with improvement of the sustained release or subcellular targeting.

Keys Words:
Irinotecan, Doxorubicin, Paclitaxel, beads, nanocapsules, glioma, radiosensitization.