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Decreased circulation time offsets increased efficacy of PEGylated nanocarriers targeting folate receptors of glioma

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Abstract
Liposomal and other nanocarrier based drug delivery vehicles can localize to tumours through passive and/or active targeting. Passively targeted liposomal nanocarriers accumulate in tumours via 'leaky' vasculature through the enhanced permeability and retention (EPR) effect. Passive accumulation depends upon the circulation time and the degree of tumour vessel 'leakiness'. After extravasation, actively targeted liposomal nanocarriers efficiently deliver their payload by receptor-mediated uptake. However, incorporation of targeting moieties can compromise circulation time in the blood due to recognition and clearance by the reticuloendothelial system, decreasing passive accumulation. Here, we compare the efficacy of passively targeted doxorubicin-loaded PEGylated liposomal nanocarriers to that of actively targeted liposomal nanocarriers in a rat 9L brain tumour model. Although folate receptor (FR)-targeted liposomal nanocarriers had significantly reduced blood circulation time compared to PEGylated liposomal nanocarriers; intratumoural drug concentrations both at 20 and 50 h after administration were equal for both treatments. Both treatments significantly increased tumour inoculated animal survival by 60–80% compared to non-treated controls, but no difference in survival was observed between FR-targeted and passively targeted nanocarriers. Therefore, alternate approaches allowing for active targeting without compromising circulation time may be important for fully realizing the benefits of receptor-mediated active targeting of gliomas.

1. Introduction

In the past few decades, liposomal nanocarriers have been extensively investigated as drug carriers for cancer therapy and have been found to offer many benefits when utilized for drug delivery. One of the major benefits of PEGylated liposomal nanocarriers is their ability to evade the reticuloendothelial system (RES) and extend the circulation time of encapsulated drugs in the bloodstream. PEG chains on the outer leaflet of the liposomal bilayer are thought to provide a steric barrier to opsonin binding resulting in RES evasion [1–8]. Prolonged circulation in the bloodstream results in enhanced extravasation at sites exhibiting increased vasculature permeability [9–12]. For this reason, liposomal nanocarriers have shown increasing promise as drug delivery

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vehicles with the characteristic ability to passively accumulate in tumours and areas of injury.

Circulation time is an important factor for nanocarrier therapy of tumours and consequently much effort has gone into designing and optimizing liposomal nanocarriers to exhibit prolonged circulation times. Numerous studies have examined the effects of liposome size [13–15], charge [16], pH dependence [17], lipid composition [15, 18], cholesterol percentage [19], polymer incorporation [11], and degree of phospholipid saturation [20]. The results of these studies have confirmed that prolonged circulation is vital for passive targeting of liposomal nanocarriers at sites with 'leaky' vasculature. Passive targeting is achieved when liposomal nanocarriers are formulated to evade the RES and subsequently accumulate in areas with characteristic 'leaky vasculature', such as tumour sites or sites of injury. Drainage in tumours is typically limited, and the liposomal nanocarriers are retained at the site. This well-documented phenomenon has been designated the enhanced permeability and retention (EPR) effect and results in passive accumulation of liposomal nanocarriers at sites with compromised vasculature [9–12].

To further increase the efficacy of liposomal nanocarriers that reach the sites of interest, active targeting of drugs through the incorporation of targeting moieties on the exterior of nanocarriers has been explored. Active targeting offers many benefits including decreased side effects to reduced accumulation in non-target organs [21–23]. When targeting moieties are included in the liposomal formulation, these carriers can be made to bind specifically to target cells and/or accumulate in areas of interest [24–28]. Several studies have examined the use of ligands [29–33], small peptides [34, 35], or antibodies [26, 36–38] to target over-expressed agents present on or around target cells.

Although active targeting of liposomal nanocarriers to tumours through the incorporation of targeting moieties has shown great promise *in vitro*, numerous *in vivo* studies utilizing targeting ligands designed to direct formulations to extravascular sites have not been as successful. FR-targeted studies, in particular, have shown very little success on solid tumours *in vivo* [39, 40]. Limited success has been achieved with FR targeting in ascitic tumour models, but the increase in efficacy has been attributed to the fact that the tumours are disperse and do not limit drug diffusion or receptor binding [39, 41–44]. It has been suggested that solid tumours are more difficult to treat because drug delivery is impeded by the local tumour environment. High interstitial pressures characteristic of tumours are known to prohibit transport of liposomal drugs beyond the perivascular space [45]. We suggest, and this study confirms, that while limited transport is a viable culprit, decreased circulating levels of drug also plays a substantial role in diminishing the success of these formulations. In fact, when circulation is not compromised, for example in immune-deficient animals, FR-targeted liposomal treatments have demonstrated greater success on solid tumours [46]. In addition, a study utilizing an *in vivo* adoptive tumour growth assay, which is unaffected by pharmacokinetic parameters, exhibited a distinct advantage of FR-targeted formulations over non-targeted in inhibiting tumour growth [47].

Therefore, long circulation times and receptor-targeted uptake comprise the two major facets of liposomal drug delivery, passive targeting and active targeting. Numerous studies have successfully documented the ability of targeted liposomal nanocarriers to bind specifically to target cells *in vitro* [29–31, 33, 47]; however, others have shown that the addition of targeting moieties often has a detrimental impact on RES evasion *in vivo* even when passive targeting methods, such as the inclusion of PEG, are used in combination [34, 39, 46, 48–50]. The liposomal drug delivery strategy to achieve the highest drug accumulation at the target site with limited uptake by non-target organs would ideally incorporate both active and passive methods of targeting where each method of delivery retains optimal performance.

In the present study, we attempted to specifically target a chemotherapeutic drug, doxorubicin, to a folate receptor (FR) over-expressing intracranial tumour in immunocompetent rats using a liposomal delivery vehicle. We chose a brain tumour model to perform these studies as it represents an invasive, non-localized tumour that is difficult to treat by conventional methods such as surgical resection and/or radiation therapy. These tumours typically exhibit projections into the brain demonstrating the diffuse and invasive nature of the disease. The standard chemotherapeutic agents for brain tumours, nitrosoureas, have not been very effective as single agents and have failed to significantly improve survival times of patients compared with radiotherapy alone [51–54]. For this reason, targeted chemotherapeutics are desirable. We have chosen to target a chemotherapeutic, doxorubicin, to glioma using liposomal nanocarriers. Doxorubicin was selected for encapsulation since it is fluorescent, allowing for ease of detection, and it can be actively and stably loaded into liposomal nanocarriers, allowing for large drug payloads. In addition, doxorubicin has been shown to be more potent than nitrosoureas against glioma cells *in vitro* [55]. Liposomal doxorubicin has been investigated by several groups in the treatment of gliomas with promising results [56–58]. FR-targeted formulations have been studied extensively and represent an ideal targeting system [31, 39–44, 46, 49, 59–61]. Numerous tumours have been identified that over-express FR, and FR-targeted liposomal nanocarriers have been shown to actively bind these malignant cells and subsequently undergo endocytosis. Cellular uptake of FR-targeted liposomal nanocarriers has been shown to be dependent on the presence of adjacent PEG chains. Gabizon *et al* demonstrated that cell association of FR-targeted liposomal nanocarriers due to FR binding was completely inhibited when adjacent PEG chains were the same length as the folate-bearing PEG chains. Lengthening the folate-bearing PEG chains allowed for a dramatic increase in binding of FR-targeted liposomal nanocarriers to target cells [62]. In previous studies, we have investigated the liposomal delivery of doxorubicin to glioma cells *in vitro* utilizing FR as a target. We have previously demonstrated that preferential uptake of doxorubicin by glioma cells can be achieved using FR-targeted liposomal nanocarriers containing an optimal number of targeting ligands [29]. For this study, folate-PEG conjugates were inserted into the bilayer of liposomal nanocarriers carrying doxorubicin to facilitate targeting to FR. We investigated whether this technique increases the doxorubicin dosage received by tumour cells while reducing non-specific delivery to tissues, which do not over-express FR receptor. In addition to dosage

obtained at the tumour site, we evaluated delivery to non-target organs, clearance times, and overall increase in survival of tumour inoculated rodents relative to non-targeted or untreated control rodents. Ultimately, we examined the effect of converting a passively targeted formulation to actively targeted to determine whether the accompanying negative impact on passive accumulation offsets the benefit of active targeting. The resultant data emphasize the need to consider the effects, both beneficial and detrimental, of active targeting when formulating targeted liposomal nanocarriers to tumours.

2. Materials and methods

2.1. Materials

A 9L glioma cell line was received as a generous donation from the Neurosurgery Tissue Bank at UCSF. Minimal essential medium containing Earle's balanced salt solution (MEM/EBSS) was purchased from Hyclone (Logan, UT). Gentamicin (50 mg ml^{-1}), fetal bovine serum (FBS), and Leibovitz's L-15 medium were obtained from Gibco (Carlsbad, CA). Trypsin-EDTA (0.05% trypsin, 0.53 mM EDTA) in Hanks' balanced salt solution was purchased from Mediatech (Herndon, VA). Heparin (1000 USP units ml^{-1}), isoflurane, and doxorubicin were obtained from Baxter Healthcare (Deerfield, IL). Ketamine (100 mg ml^{-1}) was purchased from Fort Dodge Laboratories (Madison, NJ). Marcaine (0.5%) was obtained from Abbott Laboratories (Abbott Park, IL). Flunixin meglumine was purchased from Phoenix Scientific (San Marcos, CA). Xylazine (100 mg ml^{-1}) was purchased from The Butler Company (Dublin, OH). Acetylpromazine (10 mg ml^{-1}) was obtained from Boehringer Ingelheim (Ingelheim, Germany). Trifluoroacetic acid (TFA) and triethylamine (TEA) were obtained from Fisher Scientific (Pittsburgh, PA). Cholesterol, paraformaldehyde, and Triton X-100 were purchased from Sigma (St Louis, MO). 1,2-distearoyl-*sn*-glycerophospho-choline (DSPC), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE), and 1,2-distearoyl-*sn*-glycerophosphoethanolamine poly(ethylene glycol)$_{2000}$ (DSPE-PEG$_{2000}$) were obtained from Avanti Polar Lipids (Birmingham, AL). t-Boc-HN-PEG$_{3350}$-succinimidyl propionate (t-Boc-HN-PEG$_{3350}$-SPA) was obtained from Shearwater Polymers (San Carlos, CA). A monoclonal antibody to nestin (mAb 353, IgG1) was purchased from Chemicon (Temecula, CA). Dialysis tubing (10 000 and 100 000 molecular weight cut-off) was purchased from Spectra/Por (Dominguez, CA). All animals were purchased from Harlan (Indianapolis, IN) and maintained on a folic acid deficient diet (<0.05 ppm) containing 1% succinylsulfathiozole obtained from Purina TestDiet (Richmond, IN). A stereotaxic frame was purchased from Kopf Instruments (Tujunga, CA) and utilized for tumour inoculation surgeries.

2.2. Liposome formulation

Liposomal nanocarriers were formulated using methods similar to those described elsewhere [63, 64]. In brief, a 62:35:3 molar ratio of DSPC:cholesterol:DSPE-PEG$_{2000}$ was dissolved in ethanol (60 °C) and then hydrated with 400 mM ammonium sulfate buffer. The addition of 3% DSPE-PEG$_{2000}$ was to enable RES evasion. The solution was extruded five times through a 0.2 μm filter and then ten times through a 0.1 μm filter using a 10 ml Lipex Thermoline extruder (Northern Lipids, Vancouver, British Columbia, Canada) at 60 °C. Liposomal nanocarriers were then dialysed against decreasing concentrations of sodium chloride buffer to remove ethanol and establish an ammonium sulfate gradient used to facilitate doxorubicin loading. The average diameter of extruded liposomal nanocarriers was verified by dynamic light scattering (Brookhaven Instruments Corporation, Holtsville, NY) and determined to be approximately 110–115 nm.

2.3. DSPE-PEG$_{3350}$-folate conjugate synthesis

Since folate was to be utilized as a targeting ligand, it was necessary to formulate a DSPE-PEG$_{3350}$-folate conjugate to allow insertion into the liposomal bilayer. A longer PEG chain (PEG$_{3350}$) than those used to confer steric stabilization (PEG$_{2000}$) was utilized to avoid the documented interference with FR binding when shorter PEG chains are used [62]. First, a DSPE-PEG$_{3350}$-amine was synthesized according to methods described elsewhere [65]. Briefly, t-Boc-HN-PEG$_{3350}$-SPA was dissolved in chloroform and mixed with DSPE followed by TEA (~1:1:3 molar ratio). The solution was heated to 60 °C for 5 min and then mixed overnight at room temperature. The chloroform was rotary evaporated, and the residue was taken up with acetonitrile. The unreacted DSPE was precipitated by storing the mixture at 4 °C for 6 h. The solution was then centrifuged to remove the unreacted DSPE, evaporated, and dried over P_2O_5 under vacuum. A 10% solution of TFA in methylene chloride was added to the DSPE-PEG$_{3350}$-t-Boc product and mixed at 0 °C for 2 h. The mixture was then washed four times with chloroform to remove the TFA and rehydrated with water. The solution was dialysed (100 000 MWCO) against water to remove unreacted Boc-PEG$_{3350}$-amine and then lyophilized to yield the DSPE-PEG$_{3350}$-amine product.

Next, the DSPE-PEG$_{3350}$-folate conjugate was formed by previously described methods [29, 62]. Briefly, 36.8 mg of folate was dissolved in 1.415 ml of dry DMSO before adding 190 mg of DSPE-PEG$_{3350}$-amine, 600 μl of pyridine, and 46 mg of dicyclohexyl carbodiimide (DCC). The mixture was allowed to react for 4 h at room temperature. The solution was rotary evaporated to remove pyridine and rehydrated with 17.5 ml water. Insoluble by-products were removed by centrifugation at 10 000*g*. The supernatant was dialysed (100 000 MWCO) twice against 2 l 50 mM NaCl and three times against 2 l water. The retentate was then lyophilized to yield the final product, which was analysed by thin-layer chromatography, ^{1}H NMR, and mass spectroscopy. R_f = 0.49 in 1.48 N ammonium hydroxide. ^{1}H NMR ($CDCl_3$ solvent): DPPE (0.84 ppm (t), 1.2, 1.5(d), 2.25(d), 2.9(t), 3.1(t), 5.04(m)), PEG (3.3 ppm), and folic acid (1.91, 2.03, 2.3(t), 4.33(m), 4.48(d), 6.5(d), 6.93(t), 7.64(d), 8.12(d), 8.6(s)). MW = 3144 Da.

2.4. DSPE-PEG$_{3350}$-folate insertion into preformed liposomal nanocarriers

Folate conjugates were inserted into preformed liposomal nanocarriers to create FR-targeted liposomal formulations.

Non-targeted liposomal nanocarriers did not receive conjugates for insertion. Conjugate insertion was performed according to methods previously described [29, 66]. Briefly, DSPE-PEG_{3350}-folate conjugates were micellized by dissolving in DMSO (60 °C) to a concentration of 28 mM and then diluting 1:10 with water for a final concentration of 2.8 mM conjugate in 10% DMSO. Micelles were dialysed (10 000 MWCO) twice against 1 l water to remove DMSO. Folate content of the retentate was determined by measuring UV absorbance at 285 nm wavelength of micelles lysed in 10% SDS using a UV–visible spectrophotometer (Shimadzu Scientific Instruments Model 1601, Columbia, MD). Folate conjugate micelles were then mixed with liposomal nanocarriers to achieve a concentration of 0.15% of the total lipid formulation. Our previous *in vitro* studies have demonstrated that maximum differentiation in drug uptake between malignant and cortical cells was achieved with 0.2% folate conjugate insertion in the absence of adjacent DSPE-PEG_{2000} chains [29]. 0.15% folate conjugate was inserted to generate the FR-targeted formulations for these *in vivo* studies since the insertion of higher numbers was partially hindered by adjacent DSPE-PEG_{2000} chains and this number allowed for consistent insertion efficiencies. Previous *in vitro* studies from our lab have also demonstrated that FR-targeted formulations containing as little as 0.05% folate conjugate bind efficiently to malignant cells, consequently this reduction in targeting ligand insertion would still promote targeting to tumour cells [29]. The micelle/liposome mixture was then heated to 60 °C for 1 h to allow insertion. Afterwards, the liposomal nanocarriers were cooled on ice and then dialysed (100 000 MWCO) to remove any ammonium sulfate or unincorporated folate conjugates from the external phase of the liposomal nanocarriers. To verify adequate insertion, the folate content in the liposomal formulation was then analysed by measuring the UV absorbance at 285 nm after lysing the liposomal nanocarriers with 10% SDS.

2.5. Remote loading of doxorubicin

Doxorubicin was loaded into the liposomal nanocarriers using an ammonium sulfate gradient as previously described [64]. In brief, liposomal nanocarriers were mixed with doxorubicin reconstituted in PBS (15 mg ml^{-1}) at a ratio of 0.16 mg doxorubicin to 1 mg of lipid and heated to 60 °C for 1 h. The liposomal nanocarriers were then immediately cooled on ice and subsequently dialysed to remove any remaining doxorubicin. The formulations were sterilized by passing through a 0.2 μm filter. Final doxorubicin content was assessed by lysing the liposomal nanocarriers with 5% Triton X-100 at 60 °C and measuring the UV absorbance at 480 nm.

2.6. Plasma clearance

In an effort to separate the effects of RES clearance and extravasation into tumour, these studies were performed in animals without tumours. Animals were given an intravenous (IV) injection of either non-targeted ($n = 5$) or FR-targeted liposomal doxorubicin ($n = 5$) via tail vein (10 mg kg^{-1} doxorubicin; ~60 mg kg^{-1} lipid). Blood was collected from the orbital sinus immediately before injection and at 1, 3, 12, 16, 22, 48, and 92 h after injection. Plasma was isolated by centrifugation (2200g, 15 min). Liposomal nanocarriers were lysed by treating with 5% Triton X-100 and heating to 60 °C for 20 min. To accurately detect low levels of doxorubicin, fluorescent readings were obtained. Total doxorubicin content of each sample was analysed ($\lambda_{ex} = 485$, $\lambda_{em} = 590$) using a fluorescence spectrometer (BIO-TEK, Synergy HT, Winooski, VT). Plasma samples obtained immediately prior to injection were used to correct for background fluorescence.

2.7. 9L glioma cell culture

A 9L glioma cell line was maintained in MEM/EBSS medium supplemented with 10% fetal bovine serum and 0.05 mg ml^{-1} gentamicin. Cells were passaged by trypsinization and washed with growth medium. Prior to implantation, cells were resuspended in serum-free Leibovitz's L-15 medium to a concentration of 2×10^8 cells ml^{-1}.

2.8. Tumour inoculation

A rat glioma model was established by surgically implanting 2×10^6 9L glioma cells into the frontal lobe of 11–12 week old male Fisher 344 rats. All procedures were conducted under a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at Georgia Institute of Technology. Animals were fed a folate-free diet containing 1% succinylsulfathiozole for approximately 18 days prior to surgery. Folate was eliminated from the diet in an attempt to prevent competitive binding of dietary folate to the folate receptors on tumour cells and to avoid possible down regulation of tumour FR after implantation. Although other studies have shown exclusion of dietary folate to have no effect on tumour uptake of exogenous folate, a succinylsulfathiozole supplement was not utilized to eliminate production of folate by the enteric microflora [39]. In addition, a study utilizing J6456 lymphoma *in vivo* demonstrated a quick down regulation of tumour FR expression when animals were kept on a normal folate-enriched diet [46]. Therefore, in our study, animals were maintained on the folate deficient diet for a minimum of 3 weeks prior to treatment as this has been proven adequate to sufficiently lower folate concentrations to a level comparable to that of humans [46]. During surgery, anaesthesia was maintained through the administration of 2–3% inhalant isoflurane. The incision site was shaved and the animal mounted in a stereotaxic frame. The scalp was opened to expose the skull, and a burr hole was drilled 2 mm anterior and 2 mm lateral to the bregma. 2×10^6 9L glioma cells in 10 μl of Leibovitz's L-15 medium were slowly injected into the frontal lobe through a 21-gauge needle at a depth of 3 mm below the brain surface. The burr hole was then sealed with bone wax, and the scalp was sutured closed. Animals received 5 ml Lactated Ringer's solution through intraperitoneal (IP) injection and a subcutaneous injection of 0.5% marcaine at the wound site. Flunixin meglumine (2.5 mg kg^{-1}) was administered through an intramuscular injection to alleviate pain as needed.

2.9. Immunohistochemistry for characterization of angiogenesis

To ensure that the injection of liposomal nanocarriers would occur after the onset of angiogenesis, explanted brains

were examined for new blood vessel formation. Four days after tumour inoculation, rats were anesthetized by an IP injection of 50, 10 and 1.67 mg kg^{-1} respectively of ketamine/xylazine/acetylpromazine and perfused with phosphate-buffered saline (PBS) containing heparin (1000 units l^{-1}) followed by 4% paraformaldehyde in PBS. Brains were explanted and stored in 4% paraformaldehyde for approximately 24 h prior to being embedded in paraffin. Paraffin embedded tissues were sliced into 5 μm sections using a rotary microtome. Representative sections containing tumour were immunostained for nestin, a marker of angiogenesis. In brief, sections were deparaffinized and rehydrated using xylene and a graded series of alcohols and then washed in PBS. Endogenous peroxidase was blocked by treating with 1% H_2O_2 in PBS, and the sections were treated with proteinase K (1 μg ml^{-1}) for antigen retrieval. After washing with PBS and permeabilizing with 0.5% saponin in PBS, sections were exposed to a mouse monoclonal antibody to nestin (1 μg ml^{-1}) in PBS containing 1% horse serum for 1 h. Sections were then washed twice with PBS before applying the biotinylated secondary antibody in 0.5% saponin in 1% normal horse serum in PBS for 1 h at room temperature. Slides were washed with PBS and incubated for 45 min with Vectastain Elite ABC Reagent. Afterwards, sections were washed with PBS and then sterile deionized water and exposed to the vector DAB substrate (0.067% in tris-buffered saline with 0.024% H_2O_2). The reaction was terminated by washing with PBS, and the sections were then counterstained with Harris' haematoxylin.

2.10. Biodistribution in tumour inoculated animals

Thirteen days after glioma inoculation, when tumour was deemed large enough for explantation, an orbital blood sample was collected from each animal prior to treating with either a saline sham, non-targeted 'Stealth' (20 h: $n = 8$; 50 h: $n = 8$), or FR-targeted (20 h: $n = 7$; 50 h: $n = 10$) liposomal doxorubicin IV injection (10 mg kg^{-1} doxorubicin; ~60 mg kg^{-1} lipid) via tail vein. At each of two designated time points following injection (20 or 50 h), doxorubicin biodistribution was assessed. Numerous investigators have reported liposomal accumulation in tumour to peak around 48 h [39, 67, 68], which lead to the selection of the 50 h time point for this study. The earlier time point (20 h) was selected since it has been reported that FR-targeted liposomal nanocarriers may have an increased accumulation in tumour compared to non-targeted liposomal nanocarriers at earlier time points [39]. At each time point after doxorubicin administration, animals were anesthetized with an IP injection of 50, 10 and 1.67 mg kg^{-1} respectively of ketamine/xylazine/acetylpromazine, and a cardiac blood sample was obtained. Animals were then perfused with heparinized PBS (1000 units l^{-1}) to remove the blood. The spleen, brain, heart, lungs, liver, and kidneys were explanted, washed with PBS, and blotted dry. Tumour, identified by discoloration and variation in tissue texture, was dissected from the brain using a dissecting microscope at 7× magnification. Organs were weighed and frozen at −20 °C until ready to be processed. Plasma was isolated from each cardiac blood sample obtained prior to perfusion by centrifuging at 2200g for 15 min. Plasma samples were stored at −20 °C until ready to be analysed. Doxorubicin was extracted from plasma and tissue samples in a manner similar to that described elsewhere [69]. Plasma was diluted 1:4 with water. Organs were homogenized in distilled, deionized water (20% wt/vol) using a Polytron Homogenizer (Brinkmann Instruments, Westbury, NY). Homogenates and 25% plasma samples (200 μl) were mixed with 100 μl of 10% Triton X-100, 200 μl of water, and 1500 μl of acidified isopropanol (0.75N HCl). Mixtures were stored overnight at −20 °C to extract the drug and then warmed to room temperature and vortexed for 5 min. The samples were then centrifuged at 15 000g for 20 min. Fluorescence of supernatants were analysed to determine doxorubicin content ($\lambda_{ex} = 485$, $\lambda_{em} = 590$). Organ samples from an animal treated with a saline sham IV injection and plasma samples obtained prior to doxorubicin injection were used to correct for background fluorescence.

2.11. Survival studies

Four days after tumour inoculation, animals were treated with either a saline sham ($n = 5$), non-targeted 'Stealth' ($n = 6$), or FR-targeted ($n = 6$) liposomal doxorubicin IV injection (10 mg kg^{-1} doxorubicin; ~60 mg kg^{-1} lipid) via tail vein. Equivalent volumes of 0.9% sterile saline solution were administered to animals receiving sham injections. Tumour growth was allowed to progress until the animal showed signs of morbidity, at which point, interventional euthanasia was administered. Time of death was determined to be the following day.

3. Results

3.1. Plasma clearance studies

Analysis of doxorubicin in plasma samples obtained from treated rats revealed that the insertion of 0.15% folate conjugates into liposomal nanocarriers resulted in accelerated clearance from blood plasma (figure 1). ANOVA revealed that the plasma clearance was significantly different between the two formulations ($p < 0.001$). Within the first hour, the rapid reduction in circulating FR-targeted liposomal doxorubicin resulted in a 20% difference in plasma levels of FR-targeted doxorubicin compared to non-targeted. Circulation data were fitted to bi-exponential curves. Areas under the curves and plasma half-lives were determined for each formulation and are reported in figure 1. The incorporation of 0.15% folate conjugates resulted in a 41.8% reduction in AUC. Formulations containing 0.2% folate conjugates were also investigated and found to exhibit a further decrease in circulation times demonstrated by a 61.9% reduction in AUC compared to non-targeted liposomal doxorubicin (data not shown).

3.2. Tumour growth curve

A growth curve was established to record tumour progression over time in untreated rats (figure 2). The growth curve data was used to determine inter-animal variability in tumour growth and to verify our ability to consistently inoculate tumour. Tumour volume was shown to increase exponentially with a doubling time of 1.7 days for the intracranial 9L tumour model. Tumours were reliably produced in every animal

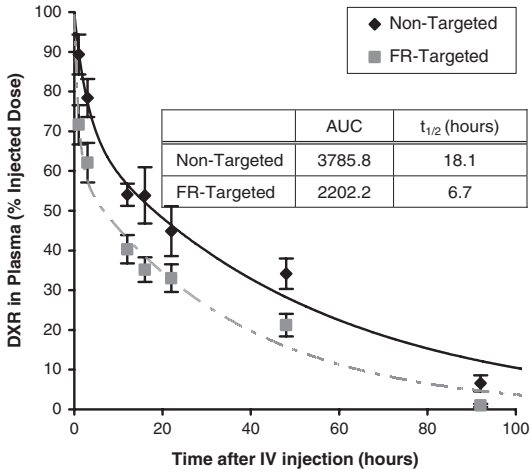

Figure 1. Plasma clearance of liposomal doxorubicin formulations. Accelerated clearance of FR-targeted liposomal formulations was exhibited by the rapid decrease of doxorubicin in the blood following an IV injection of 10 mg kg^{-1} liposomal doxorubicin. Plasma clearance of FR-targeted formulations was significantly higher than clearance of non-targeted liposomal nanocarriers as determined by ANOVA ($p < 0.001$). Both formulations included 3% DSPE-PEG_{2000} to promote RES evasion. Blood samples were collected from the orbital sinus at various time points, and drug concentration was determined by fluorometry. Error bars represent standard error of means. Areas under the curves and plasma half-lives were calculated for each formulation and are reported in the inset table.

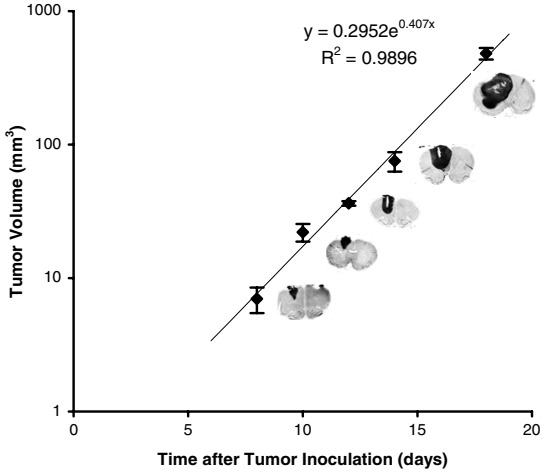

Figure 2. 9L glioma growth curve in Fisher 344 rats. Tumour volumes were determined through histological analysis. Representative slices stained with cresyl violet from brains explanted at each time point show tumour cross-sections (dark areas). Data is fitted to an exponential curve. Error bars represent standard error of means.

following stereotaxic implantation. Histological examination revealed that the tumours were infiltrative into normal brain tissue (data not shown).

3.3. Nestin expression

Angiogenesis is necessary for effective IV delivery of drug to tumours. For brain tumours, in particular, the formation of new blood vessels is critical because it disrupts the blood–brain barrier. Therefore, we performed immunohistochemistry to determine the onset of angiogenesis in our intracranial tumour model. Immunohistochemistry results confirmed that angiogenesis initiates as early as 4 days following tumour inoculation (figure 3). Nestin, an intermediate filament protein expressed by neuroepithelial stem cells, was present in the tumour at this time point indicating the formation of new microvessels (figures 3(b)–(d)). This protein was not detected in normal brain tissue (figure 3(a)). The presence of new vessels 4 days after tumour inoculation in our model makes liposomal drug delivery to tumour feasible at this time point. Since treatments are typically most effective at an early time point, day four was chosen to be the treatment day for survival studies.

3.4. Organ distribution studies

Organ analysis for doxorubicin content showed that the majority of drug was cleared by the liver and spleen for both formulations (figure 4). RES saturation is unlikely since the lipid levels did not exceed those reported to cause saturation [70]. In addition, we did not observe an acceleration of clearance as the plasma levels dropped, which would have been indicative of RES saturation. Doxorubicin levels in the kidneys were comparable between the two different formulations. Plasma doxorubicin levels, however, were significantly different between the two formulations at each time point. FR-targeted formulations showed lower plasma levels compared to non-targeted formulations at both 20 and 50 h confirming the data obtained from our plasma clearance study. Plasma doxorubicin levels decreased significantly over time for both formulations. Spleen doxorubicin levels also decreased over time, however, the reduction was only statistically significant for FR-targeted formulations as determined by Student's *t*-test.

Tumour doxorubicin levels were not significantly different between the two formulations at each time point despite the significantly lower amount of FR-targeted doxorubicin in the plasma. Doxorubicin tumour levels for non-targeted formulations decreased 6.5% from 20 to 50 h, which was accompanied by a 27% reduction in plasma levels. In contrast, a 3% increase in tumour levels was observed over the same time period for FR-targeted formulations despite the corresponding 65% reduction in plasma doxorubicin levels. Both formulations exhibited higher doxorubicin content in the tumour compared to normal brain tissue at 20 and 50 h, however, elevated doxorubicin levels in tumours were only statistically significant at the 50 h time point.

3.5. Survival studies

The therapeutic effect of the non-targeted and FR-targeted treatments was determined by comparing the respective survival times in response to treatment type (figure 5). Treatments were administered IV at a doxorubicin dosage of 10 mg kg^{-1} four days after tumour inoculation when new blood vessels have begun to emerge. Equivalent volumes of saline were administered for untreated animals. The data show a statistically significant increase in survival time for both non-targeted ($p = 0.004$) and FR-targeted ($p = 0.01$)

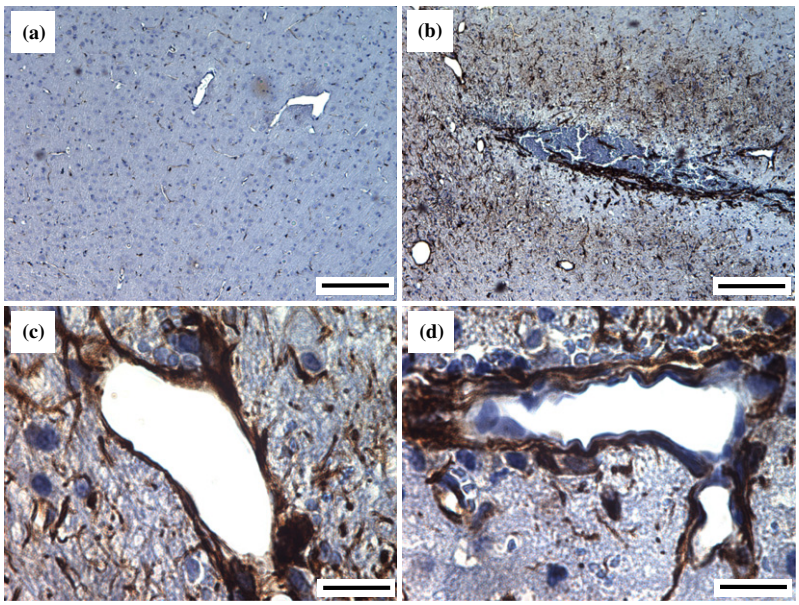

Figure 3. Nestin expression in a normal brain and 9L glioma tumour. Immunohistochemical analysis reveals expression of nestin, a marker for angiogenesis, in a tumour 4 days after inoculation. Brown horseradish peroxidase marks the location of nestin in the fixed tissue. (a) Normal brain tissue serves as a negative control. (b) A tumour section obtained from Fisher 344 rat 4 days after intracranial 9L glioma tumour inoculation exhibiting elevated expression of nestin. The scale bars represent 200 μm. (c), (d) magnifications of the tumour image (b) displaying nestin staining along microvessel walls. The scale bars represent 20 μm.

treatments when compared to a saline sham injection as determined by Student's *t*-test. However, there was not a significant difference in survival between the non-targeted and FR-targeted liposomal treatments.

4. Discussion

The present study was designed to explore the relative benefits of actively targeted FR compared to passively targeted 'Stealth' liposomal nanocarriers. Numerous *in vitro* studies have reported gains in targeting efficiency of liposomal nanocarriers to tumour cells through the inclusion of folate [29, 31, 41–44, 46, 49, 71]. While in this study tumour accumulation and survival were no different when compared to passively targeted Stealth liposomal nanocarriers, the fact that this occurred in spite of significantly compromised circulation time points to the advantages of active targeting on tumour states. We hypothesize that the increase in clearance exhibited by FR-targeted liposomal nanocarriers was due to recognition of folate by the RES. Exposure of folate on the liposomal surface is believed to elicit an interaction between liposomal nanocarriers and factors responsible for RES clearance. This interaction occurs even though PEG is included in the liposomal formulation. Increasing the numbers of PEG chains beyond 3% in the liposomal formulation was not attempted because we have previously determined that increasing the number of PEG molecules tends to hinder insertion of folate-PEG_{3350}-DSPE conjugates into the liposome bilayer and might compromise the access of folate to its receptor on tumour cells. The observed marked reduction in circulation times of FR-targeted formulations, while significant, was not as dramatic as that reported for formulations utilizing antibodies as targeting moieties [37, 50], but was slightly higher than that reported in a similar study utilizing a higher lipid dose in mice [39].

Survival studies verified that the inclusion of folate in the liposomal formulations did not extend survival beyond that achieved by passively targeted 'Stealth' liposomal nanocarriers. While this is consistent with the equivalent accumulation of doxorubicin in tumours in both cases, it is also possible that survival as the ultimate endpoint of this study is not sufficiently sensitive to assess differences in behaviour of single doses. It is possible that because this tumour model exhibits exponential growth, a single injection may not be capable of halting growth enough to cause a substantial increase in survival. Tumour growth probably recovers rapidly following a single injection of chemotherapeutic making it impossible to see an effect weeks later. Even if tumour size was reduced to a larger degree by the targeted formulations, the aggressiveness of the tumour to recur and the ease to expand within an empty void may have made it impossible to ultimately resolve a difference in survival times. Numerous studies, in fact, have demonstrated greater success in extending survival times of animals and tumour accumulation of drug when multiple treatments are administered [67, 72]. The administration of multiple treatments may have revealed a difference in efficacy between the targeted and non-targeted liposomal treatments in the current study; however, this was not attempted because our ultimate goal was to compare passively targeted and actively targeted formulations using a single treatment regimen to examine the effects of active targeting more closely.

To determine why survival times remained unaffected despite the inclusion of folate, biodistribution studies were performed to evaluate drug content in the vital organs. A significantly higher liver accumulation of FR-targeted over

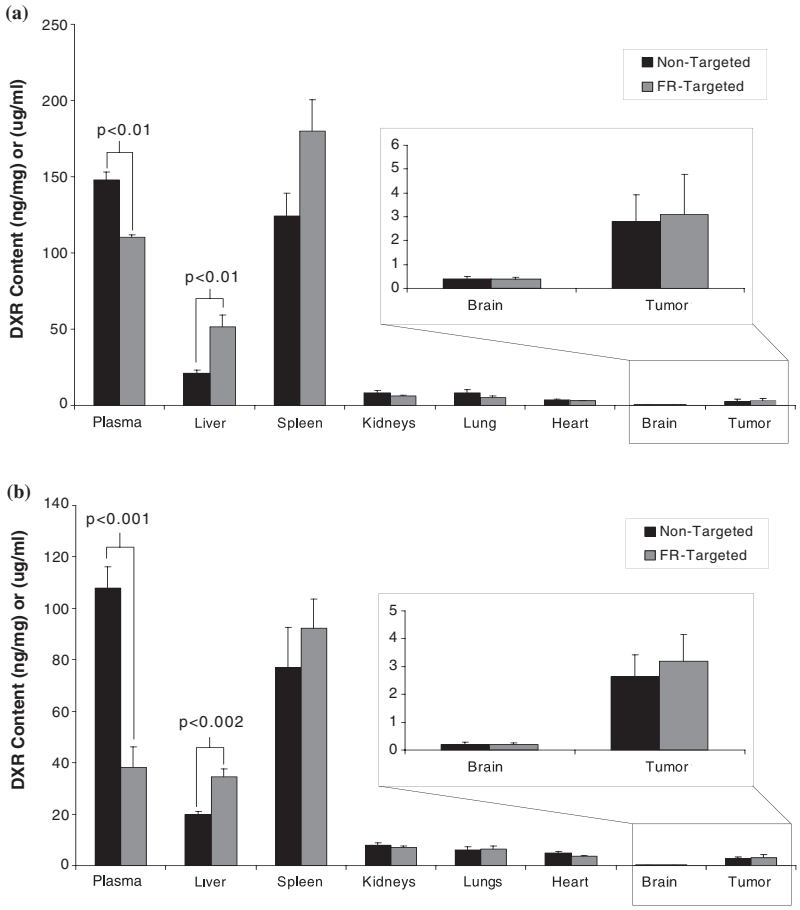

Figure 4. Biodistribution of liposomal doxorubicin formulations in Fisher 344 rats with 9L glioma tumour. Animals received 10 mg kg^{-1} doxorubicin IV in either non-targeted or FR-targeted liposomal nanocarriers. Fisher 344 rats were euthanized (a) 20 h or (b) 50 h after doxorubicin administration. Doxorubicin content in each organ (ng mg^{-1}) and within the plasma (μg ml^{-1}) was determined through fluorometric analysis. At both time points, plasma doxorubicin concentrations in animals treated with FR-targeted doxorubicin were significantly lower ($p < 0.01$, $p < 0.001$) and liver concentrations were significantly higher ($p < 0.01$, $p < 0.002$) than animals receiving non-targeted formulations (Student's t-test). The inset graphs illustrate tumour and brain doxorubicin levels. At 50 h, both groups exhibited significantly higher doxorubicin levels in tumour compared to brain ($p < 0.01$). The error bars represent standard error of means.

non-targeted liposomal nanocarriers (20 h: $p < 0.01$, 50 h: $p < 0.001$) obtained from this study suggests that the liver is primarily responsible for the accelerated plasma clearance observed for FR-targeted formulations. This is either due to specific targeting to the folate receptors on hepatic cells or a result of opsonization of FR-targeted formulations resulting in clearance through the liver. Since kidney levels were comparable for both formulations, we are confident that both formulations are equally stable and do not 'leak' doxorubicin. Therefore, significant reduction in plasma levels of FR-targeted drug compared to non-targeted observed at each time point was due to accelerated RES clearance.

Comparable amounts of drug were attained in the tumour for both formulations despite significantly lower plasma levels of FR-targeted drug at each time point. Passive accumulation of liposomal nanocarriers at pathological sites has been shown to be a function of circulation kinetics [73]. This is true up to a certain lipid dose, at which saturation of tumour with liposomal nanocarriers would occur. The fact that FR-targeted liposomal nanocarriers demonstrated decreased plasma levels suggests that tumour accumulation of doxorubicin should have been proportionately lower compared to non-targeted, however, this was not the case. Saturation of tumour with doxorubicin is an unlikely cause of this result since tumour levels continued to increase over time, and even at the maximum accumulation levels, it is estimated that only 18% of the tumour interstitial volume would have been occupied by liposomal nanocarriers. Total per cent of interstitial volume occupied by liposomal nanocarriers was calculated assuming a tumour density close to 1 g cm^{-3} and an interstitial volume fraction of 0.4 as determined by previous investigators [74].

Since it is assumed that the tumour input of doxorubicin is decreased for FR-targeted formulations due to lower circulating levels, the fact that comparable drug levels were discovered in the tumours suggests that FR-targeted formulations may have been retained to a better degree within the tumour due to the presence of the folate ligand. While we acknowledge the fact that direct evidence for active targeting and enhanced retention is not reported in this study, others have demonstrated that active targeting to cells obtained from solid mass tumours with FR-targeted liposomal formulations is achievable *in vivo* [44]. In addition, the relationship

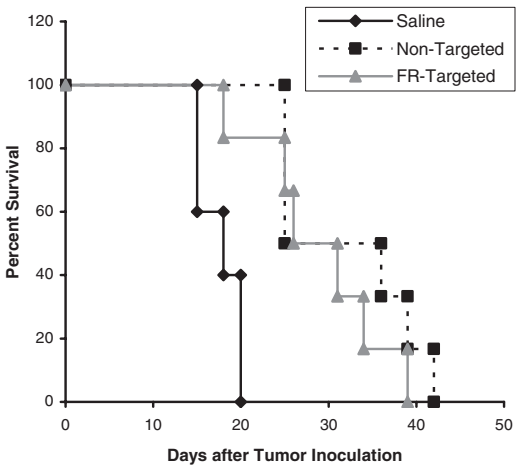

Figure 5. Survival of Fisher 344 rats with 9L glioma tumour in response to treatment. Intracranial tumour implantation occurred on day 0, and treatments were administered 4 days later. Liposomal doxorubicin was administered IV at a dose of 10 mg kg^{-1}. Animals in the saline sham group received equivalent volumes of 0.9% saline IV. Survival was monitored daily as described in section 2. Survival of animals receiving liposomal doxorubicin was significantly increased over saline treated animals ($p < 0.02$), however there was no significant difference in survival between animals receiving either non-targeted or FR-targeted liposomal doxorubicin (Student's t-test).

between circulation time and passive targeting of liposomes to tumour sites is well established [70, 73, 75]. Decreased circulating levels of liposomes have been proven to lead to decreased tumour uptake; however, this was not the case in our studies presumably due to active targeting and improved retention of targeted formulations at the tumour site. Altering the dosage of lipids (number of nanocarriers) administered was not attempted because higher dosages would have led to RES saturation and lower dosages would have resulted in a further reduction in passive targeting to tumour. We did attempt to increase the amount of targeting ligand present on the liposomes in an effort to enhance the ability to actively target tumour and overcome the loss in circulation time, however, this simply resulted in even lower circulating levels of liposomes in the bloodstream further strengthening our thesis that the presence of folate causes the accelerated clearance. Decreasing the amount of folate present on the liposomes was not attempted because our previous *in vitro* studies have indicated that this would lead to a reduction in tumour targeting [29]. These studies reinforce the central finding of this report: while active targeting and retention at the tumour site confers a major advantage of FR-targeted over non-targeted formulations, decreases in nanocarrier circulating time effectively offset the gain in drug retention resulting in comparable survival times with both treatments.

The results obtained from these experiments stress the importance of carefully considering all of the effects related to active targeting. There exists a definite need to properly balance the effects of passive and active targeting when preparing liposomal formulations. Potential therapeutic formulations must be tailored so that the benefits of active targeting are not offset by a potential decrease in circulating levels of drug. To address this need, special considerations must be made upon the inclusion of targeting moieties to not only allow for adequate drug targeting, but also ensure that time spent in circulation is not compromised.

5. Conclusions

To date, the majority of studies utilizing FR-targeted formulations have shown very modest, if any, improvements in treatment efficacy. Lack of success achieved with FR-targeted formulations has been attributed to the limitations on the transport of liposomal nanocarriers within solid tumours. We believe, however, that the inability to improve treatment efficacy with targeted liposomal nanocarriers *in vivo* may also be due, in part, to the losses in passive accumulation in tumours due to compromised circulation times associated with actively targeted formulations. This information contributes to the current understanding of how potential therapeutic formulations may be tailored to address the combined effects of drug retention in tumour and time spent in circulation. If plasma clearance issues can be resolved, the ability to specifically target chemotherapeutics to malignant brain tumours would alleviate some of the issues associated with current therapies and possibly allow for improved prognoses.

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