Masking and Triggered 'Unmasking' of Targeting Ligands Improves Liposomal Delivery to Glioma

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Statement of Purpose: Liposomal nanocarriers have been studied extensively as delivery vehicles for chemotherapeutics because liposomes are capable of increasing drug delivery to tumors due to the combination of prolonged circulation in blood and enhanced permeability and retention (EPR) at the tumor site[1]. Liposomes can also be tagged with targeting molecules to further increase targeting specificity and drug uptake by tumor. However, when targeting moieties are employed, circulation times are often decreased due to recognition by the reticulo-endothelial system (RES)[2,3], and passive accumulation to tumors is negatively affected[4]. To address this issue, we developed a liposomal system using cleavable polyethylene glycol (PEG) chains designed to prevent RES recognition of targeting ligands, thereby prolonging circulation times, while maintaining the ability to actively target tumor cells upon detachment of PEG.

Methods: A cysteine-cleavable PEG conjugate was synthesized by mixing N-Succinimidyl 3-[2-pyridyldithio]-propionamido (SPDP) and DSPE in chloroform with TEA and adding $PEG_{5000}SH$ 5 hours later. After reacting for 2 days, impurities were removed through column chromatography. Liposomes were formulated from DSPC, cholesterol, and DSPE-PEG_{2000} or DSPE-S-S-PEG_{5000}. Folate receptor targeted formulations received DSPE-PEG_{2000}-folate. Doxorubicin (DXR) was loaded via an ammonium sulfate gradient. *In vivo* plasma clearance studies were performed to determine the ability to shield liposomal targeting agents from the RES with cleavable PEG chains while in bloodstream circulation. Male Fisher 344 rats received liposomal DXR formulations i.v. Blood was collected and analyzed for DXR content to determine circulation times of each liposomal formulation. *In vitro* studies were utilized to investigate the ability to control presentation of targeting ligands on liposomes using cleavable PEG chains. Liposomal formulations were mixed with either a cysteine solution (10:1 molar ratio of cysteine:lipid) or an equivalent volume of saline for 30 minutes. Treatments were then applied to 9L glioma cells for 2 hours. For uptake analysis, cells were washed, fixed, and imaged on a confocal microscope. Cytotoxicity data was obtained by assessing cellular viability 5 days after treatment application. *In vivo* uptake of liposomes by target cells was assessed to further evaluate the performance of detachable PEG coatings on targeted liposomes. Liposomally encapsulated fluorescent dye was administered to 9L/LacZ tumor inoculated animals, and 28.5 hours later, either saline or cysteine solution was infused i.v. After 1.5 hours, animals were euthanized, and tumors were dissociated, stained for LacZ, and analyzed via flow cytometry to assess cellular association.

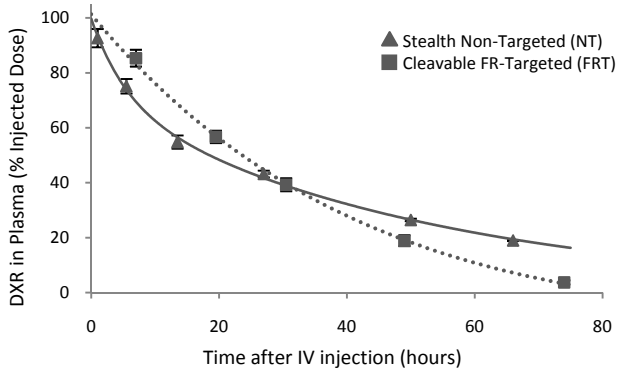

Fig 1. Inclusion of 8% DSPE–S–S–PEG_{5000} preserves circulation of FR-targeted nanocarriers.

Results: Mass spectroscopy results verified our ability to construct a conjugate of the expected molecular weight (~5824 Da). Successful cleavage of the conjugate was confirmed through TLC and HPLC. Circulation studies demonstrated that 8% DSPE-S-S-PEG_{5000} was capable of concealing folate on liposomes since circulation times of targeted formulations approached those attained with non-targeted liposomes (Fig 1). *In vitro* uptake and cytotoxicity studies verified our ability to conceal and expose folate on demand, permitting receptor mediated targeting and delivery of large drug payloads into the nucleus of target cells. Finally, *in vivo* studies conducted to analyze cellular drug uptake confirmed that delivery was significantly enhanced when tumor-inoculated animals received FR-targeted liposomes containing cleavable PEG chains followed by a cysteine infusion to expose folate (Fig 2).

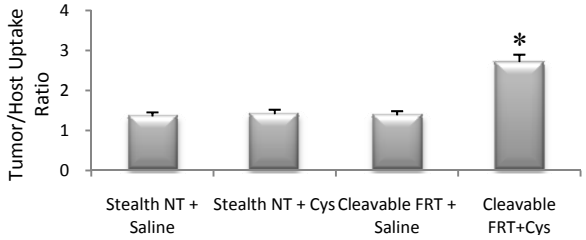

Fig 2. Uptake of liposomes is enhanced when folate is masked during circulation and ultimately exposed after extravasation into tumor.

Conclusions: Through this study, we have shown that the circulation of FR-targeted formulations may be preserved through the inclusion of PEG chains which are longer than those used to conjugate folate to the liposome. These results indicate that cleavable PEG can be used in targeted liposomal formulations to enhance efficacy of chemotherapy in the treatment of glioma by allowing for control over presentation of targeting ligands on the liposomal surface *in vivo*.

References:

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