Project Findings

The major goals of the proposal were to develop new targeting schemes to increase the specificity of liposomal localization to gliomas of the brain without sacrificing efficacy. The main idea was to exploit the co-incident overexpression of receptors on tumor cell surface by binding multiple targeting ligands in a controlled fashion on liposomal carriers, and the approach was a pattern recognition strategy to targeting rather than finding and targeting unique markers on tumor cells. Dual ligand studies were performed targeting receptors that are overexpressed on tumor cells. We had a very productive funding period with six publications resulting and the major findings from our study were the following: a) it is possible to finely tune the number of targeting ligands to differentially deliver chemotherapeutic agents to cells with higher folate receptor number (KB cells) compared to cells with lower receptor number (9L glioma); b) we can mathematically model the receptor ligand binding kinetics to predict our nanocarrier-tumor cells interactions as a function of receptor number and drug uptake; c) we can encapsulate CT and MR contrast agents within the nanocarriers to help visualize them and study pharmacokinetics; and d) dual ligand nanocarriers are indeed superior to single ligand targeting. This research was highlighted on two occasions: 1) an NCI press release, and 2) a nanotechwire news story. National Cancer Institute’s Nanonews website issued a press release on March 21, 2006, covering a paper Prof. Bellamkonda and the co-investigator on this project, Prof. Annapragada, published in the Journal of Controlled Release, “Folate targeting of drug carriers: A mathematical model.” This paper detailed a new theory about the optimal design of folate targeted nano-carriers and the NCI press release indicating its potential impact can be found at http://nano.cancer.gov/media_nanotech_news_2005-03-21c.asp. Additionally, Nanotechwire news reported on research published in another Journal of Controlled Release paper, “A Dual Ligand Approach for Enhancing Targeting Selectivity of Therapeutic Nanocarriers.” The story can be found at http://nanotechwire.com/news.asp?nid=3555.

This project also contributed to the educational development of two graduate students and four undergraduate students, who were trained with NSF support. Both of the graduate students are on track to successful careers in academia. One is currently a post-doctoral fellow in Biomedical Engineering at University of Washington, Seattle and the other is a post-doctoral fellow in BME and Radiology at Duke University.

Listed below are the publications resulting from this project:

Project Activities
We made much progress toward meeting the aims over the third funding period of this project. We continued testing selectivity of single- and dual-ligand nanocarriers and also performed studies to determine the localization of cellular doxorubicin.

Testing selectivity of single- and dual-ligand nanocarriers
To test the utility of dual-ligand liposomes, we determined the LC50 for single- and dual-ligand formulations and compared the targeting selectivity enhancement of these formulations. Targeting selectivity was defined as the ratio of the LC50 values of off-target cells (with one or no targeted receptors available; one or both receptors blocked) to the LC50 values of targeted cells (all targeted receptors available; none of the receptors blocked). Although the inverse of the normal definition of selectivity, this definition is more intuitive in regards to viability measures because larger values indicate more favorable selectivity. Fig. 1A shows that the optimal single-ligand and dual-ligand nanocarriers had much lower LC50 values than non-targeted or sub-maximal single-ligand formulations. Fig. 1B–D show the selectivity enhancement of the same formulations by comparison of target cells (bearing FR and EGFR in all cases) to off-target cells with one or neither receptor available. When off-target cells had only the EGFR available (FR blocked; Fig. 1B), the maximal efficacy single-ligand folate (~400 FA) and the dual-ligand formulations were capable of selectivity enhancement because they did not lead to toxicity in off-target cells (due to unavailability of the FR in off-target cells) while achieving toxicity in target cells. Similarly, when only the FR was available in the off-target cells (EGFR blocked; Fig. 1C), maximal efficacy single-ligand mAb225 (6 mAb) and dual-ligand formulations led to selectivity enhancement (due to unavailability of the EGFR in off-target cells). When neither FR nor EGFR was available in off-target cells (both FR and EGFR blocked; Fig. 1D), maximal efficacy single-ligand (~400 FA and 6 mAb) and dual-ligand liposomes all led to selectivity enhancement because each optimized formulation was able to achieve toxicity in target cells while sparing healthy cells.

![Fig. 1](image-url)

Fig. 1. (A) LC50 values for the various liposomal-doxorubicin formulations tested provide evidence for targeting selectivity enhancement with dual-ligand liposomes. Selectivity of the liposomal-doxorubicin formulations for (B) FR blockade of off-target cells (only EGFR available on off-target cells), (C) EGFR blockade of off-target cells (only FR available in off-target cells), or (D) FR and EGFR blockade of off-target cells (FR and EGFR unavailable in off-target cells) relative to target cells bearing both receptors. * denotes P<0.05 for selectivity greater than non-targeted liposomes by pairwise means test comparisons.
Those formulations leading to greater than a three-fold selectivity enhancement between off-target and target cells relative to non-targeted liposomes (i.e., no folic acid or mAb225 target ligands) are shown as white bars. Importantly, only the dual-ligand formulations led to selectivity enhancement relative to all three off-target cell types while still having an LC50 value near those of the single-ligand formulations (Fig. 1B–D). This indicates that, in a situation in which all of these off-target cells are present simultaneously with target cells, only the dual-ligand formulation would provide a toxic effect to the target (tumor) cells while sparing off-target (healthy) cells. That is, only dual-ligand liposomes achieve selectivity relative to all off-target cells.

**Determination of cellular doxorubicin localization by laser scanning microscopy**

The distribution of doxorubicin was studied to determine if different patterns of intracellular distribution would be observed due to the use of different types of targeting ligands. Only KB cells with both receptors available were used (i.e., no blocked receptor conditions were used in this experiment). Fixed cells were observed by laser scanning confocal microscopy after treatment with doxorubicin. Fig. 2 shows localization of doxorubicin, originally encapsulated within the liposomes, after 2 h of treatment followed by fixation and DAPI counterstain. No doxorubicin signal was detectable after 2 h of incubation with non-targeted liposomes (Fig. 2A–C). Folate-targeted liposomes (Fig. 2D–F), mAb225-targeted liposomes (Fig. 2G–I) and dual-ligand liposomes (Fig. 2J–L) all showed nuclear localization of doxorubicin after 2 h of treatment as indicated by co-localization of doxorubicin and DAPI.

![Fig. 2.](image)

Fig. 2. (A), (D), (G) and (J) show a phase contrast image overlay with doxorubicin confocal slice image in KB cells. (B), (E), (H) and (K) show a DAPI confocal counterstain image for the same cells. (C), (F), (I) and (L) show the phase/doxorubicin overlaid with DAPI. (A–C) show no doxorubicin uptake with non-targeted liposomes. Folate- (D–F), mAb225- (G–I) and dual-ligand- (J–L) targeted liposomes show nuclear localization. Scale bars denote 10 μm.
This approach does not require the identification of unique ligand–receptor pairs. Rather, the results demonstrate that when properly optimized to match the receptor profile of the target cells, common targeting ligands can be utilized. The results presented in this study demonstrate the utility of such a dual or multiple ligand approach and warrant additional study and optimization. The application of such an approach may ultimately provide the ability to tailor ligand-targeted nanocarriers to fit the profile of a particular target (tumor) system, allowing for patient-specific treatments.