

## A dual-ligand approach for enhancing targeting selectivity of therapeutic nanocarriers

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Received 13 March 2006; accepted 31 May 2006

Available online 7 June 2006

### Abstract

Conjugation of ligands to nano-scale drug carriers targeting over-expressed cell surface receptors is a promising approach for delivery of therapeutic agents to tumor cells. However, most commonly utilized ligands are directed at receptors expressed not only on target cells but also on other cells in the body, leading to unintended uptake in these off-target cells. In this study, a novel, dual-ligand approach is reported, which targets tumor cells while sparing off-target cells by exploiting the fact that tumor cells typically over-express multiple types of surface receptors. This approach was tested in the human KB cell line, which over-expresses both folate receptor (FR) and the epidermal growth factor receptor (EGFR). Liposomal nanocarriers loaded with doxorubicin and bearing controlled numbers of both folic acid and a monoclonal antibody against the EGFR were designed. Cytotoxicity was used to determine targeting selectivity of the designed carriers *in vitro* by utilizing KB cells expressing both FR and EGFR and off-target control cells in which one or both receptors were blocked. The data demonstrates that nanocarriers can be designed to achieve toxicity only when all targeted receptors are available, providing an approach to improve selectivity over current single-ligand approaches. © 2006 Elsevier B.V. All rights reserved.

**Keywords:** Drug delivery; Targeting; Cancer; Liposome; Ligand

### 1. Introduction

Tumor cells commonly over-express several cell surface receptors including epidermal growth factor receptors (EGFR) [1–3], folate receptors (FR) [4] and/or others [5,6]. Targeting ligands coupled to polymeric tethers such as poly(ethylene glycol) (PEG) [7,8] have been utilized in an effort to enhance selectivity of various nanocarriers [9–11] loaded with therapeutic agents for tumor cells (target cells) by sparing healthy cells (off-target cells) that do not express the targeted receptor [12–15].

Unfortunately, it is rare that the target receptor is exclusively over-expressed in target cells alone. Further, the presence of the number of targeting ligands utilized on drug carriers has previously been demonstrated to lead to significant drug uptake even in cells expressing relatively low numbers of the targeted receptor [16,17], resulting in unintended toxicity in the off-target cells.

In this study, a novel approach to enhance targeting selectivity of drug-loaded nanocarriers is reported. Dual-ligand liposomes simultaneously targeting multiple receptors were designed to reduce toxicity in off-target cells. This strategy exploits the fact that tumor cells typically over-express not one, but several types of surface receptors. Using doxorubicin as a model drug, we hypothesized that utilization of low numbers of two types of targeting ligands would result in cytotoxicity in the tumor cells, but spare off-target cells presenting comparable levels of only one or none of the target receptors.

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The goals of this study were to (1) design, develop and characterize nanocarrier systems bearing proper numbers of two types of targeting ligands; (2) comparatively evaluate single- and dual-ligand targeting efficacy; and (3) study the targeting selectivity of dual-compared to single-ligand nanocarriers. Liposomal nanocarriers simultaneously or exclusively bearing folic acid (directed at the FR) or mAb225 (a monoclonal antibody directed against the EGFR) were constructed and characterized, as shown schematically in Fig. 1A. The hypothesis was tested in KB cells (target cells), which express both FR and EGFR, using cytotoxicity in these cells as one of the outcome measures.

## 2. Materials and methods

### 2.1. Production of monoclonal antibody

Hybridoma cells producing mAb225 (American Type Culture Collection, Manassas, VA) were cultured in RPMI1640 supplemented with 15% horse serum and 1% penicillin–streptomycin. Bulk quantities of purified antibody were purchased from Emory Vaccine Center Immunology Core Laboratory (Emory University, Atlanta, GA).

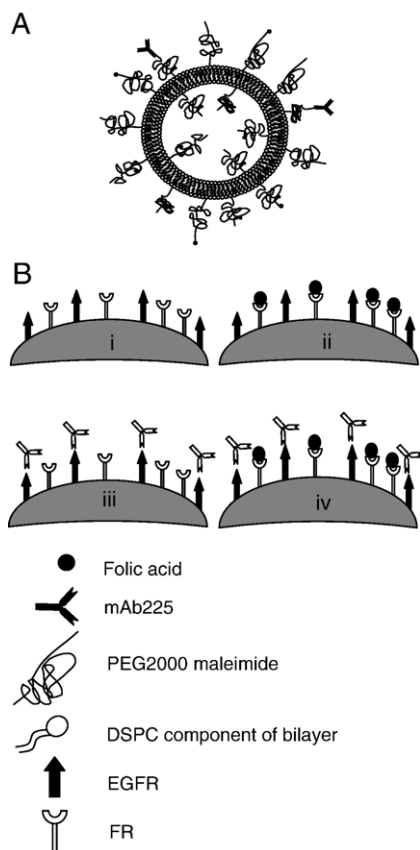


Fig. 1. (A) Schematic of dual-ligand liposomal targeting scheme that simultaneously bears both folic acid and mAb225 targeting ligands. (B) Schematic of cell culture system utilized to test dual-ligand targeting scheme. (i) KB cells (target cells) bearing both target receptors, or off-target cells with only one (ii and iii) or no receptors (iv) available.

### 2.2. Synthesis and micelle formation of DSPE-PEG3350-folate

1,2-Distearoyl-sn-glycerophosphoethanolamine (DSPE)-PEG3350-folate with PEG molecular weight of 3350 Da was synthesized by methods similar to those described previously [16,18,19]. Briefly, 0.1 mmol t-Boc-PEG3350-succinyl propionate (Nektar Therapeutics, Huntsville, AL) was reacted with DSPE overnight at room temperature. After recrystallization and washing in acetonitrile, the product was vacuum dried. t-Boc groups were then deprotected with trifluoroacetic acid. The product was washed extensively with chloroform, hydrated at a 1 mM concentration with deionized water, dialyzed and lyophilized to yield a white powder (DSPE-PEG3350-amine). DSPE-PEG3350-folate was prepared from DSPE-PEG3350-amine as described elsewhere [16,19]. Aliquots of the final product were formed into micelles at a 200  $\mu$ M concentration for incorporation into preformed liposomes [16].

### 2.3. Preparation of single-ligand folate or mAb225 liposomal nanocarriers

A 2:1:0.04 ratio of 1,2-distearoyl-sn-glycero-phosphocholine (DSPC, Genzyme, Cambridge, MA)/cholesterol (Sigma, St. Louis, MO)/DSPE-PEG2000-maleimide (Avanti Polar Lipids, Birmingham, AL), with PEG molecular weight of 2000, was dissolved in 1 mL of ethanol at 60 °C. 0.01% (mol) of fluorescent phospholipid ( $\beta$ -DPH, Invitrogen, Carlsbad, CA) was used to track phospholipid content. The lipids were hydrated with 300 mM ammonium sulfate and extruded five times through a 0.2  $\mu$ m and 10 times through a 0.1  $\mu$ m Nucleopore membrane. Liposome size was determined by dynamic light scattering (90 Plus Particle Size Analyzer, Brookhaven Instruments, Holtsville, NY). Liposomes were dialyzed against a phosphate-buffered saline solution to establish an ammonium sulfate gradient for doxorubicin loading.

Folate liposomes were prepared by adding DSPE-PEG3350-folate micelles to liposomes and heating at 60 °C for 1 h. Unincorporated micelles were removed by dialyzing the liposomes in 300,000 MWCO dialysis tubing, as described previously [16]. It is believed that incorporation of the DSPE-PEG3350-folate micelles into liposomes and partitioning of the DSPE-PEG3350-folate molecules between micellar and monomer fractions allows unincorporated DSPEPEG3350-folate to be removed with this dialysis separation.

Liposomal folic acid was determined by measuring the absorption at 285 nm and comparing to the linear region of a standard curve for folic acid. The number of folic acid molecules per liposome was determined by a ligand to phospholipid ratio, assuming 120,000 phospholipid molecules per liposome where phospholipid content was quantified by DPH fluorescence, yielding a bulk average for the number of ligands per liposome.

To fabricate mAb225 liposomes, mAb225 was thiolated with a 4:1 molar excess of 2-iminothiolane (Sigma) and 1 h reaction at room temperature [20]. Resulting mAb225-thiol was added to liposomes for overnight reaction with terminal maleimide groups on PEG. Unreacted mAb225 was removed by size

exclusion chromatography. Liposome-coupled mAb225 was determined by a modified Lowry method (DC protein assay, Bio-Rad, Hercules, CA) after lysis of liposomes with 20% SDS. The number of mAb225 ligands per liposome was determined by a ligand to phospholipid ratio as described above for folic acid. As with folic acid, the number of mAb225 represents an average of the distribution of the number of antibodies per liposome.

#### 2.4. Active loading of doxorubicin into liposomes

Following ligand incorporation or coupling, liposomes (single and dual) were loaded with doxorubicin (Henry Schein Inc., Melville, NY) via the ammonium sulfate gradient [21]. Unencapsulated doxorubicin was removed by dialysis. Loading efficiency was determined by a 480 nm absorbance reading after lysis with 5% TritonX100. Leakage of encapsulated doxorubicin under cell culture conditions (with 10% FBS) between 30 min and 3 days of incubation was determined by a dequenching [20] assay to ensure that the presence of single or dual ligands did not lead to increased leakage.

#### 2.5. Tumor cell culture

KB cells (American Type Culture Collection) were maintained in MEM/EBSS supplemented with 10% FBS and 1% penicillin–streptomycin under conditions of 5% CO<sub>2</sub> and 95% humidity.

KB cells were harvested with 0.05% trypsin/0.53 mM EDTA. Minimum essential medium/Earle's balanced salt solution (MEM/EBSS) medium was exchanged for folate free RPMI1640 (FFRPMI, Invitrogen) supplemented with 10% FBS and 1% penicillin–streptomycin. Folate-free medium with 10% FBS provides a concentration of folic acid similar to the physiological range [22]. Cells were counted with Trypan blue and a hemacytometer.

9L cells (a kind gift from the University of California at San Francisco/Neurosurgery Tissue Bank) negative for the EGFR [23] were used as a negative control to test mAb225 activity by immunohistochemistry. 9L cells were maintained as for KB cells but with gentamicin (50 mg/dL) as the antibiotic.

#### 2.6. Cytotoxicity experiments

Cytotoxicity studies were conducted by seeding KB cells (target cells) at a density of  $2 \times 10^3$  cells/well of a 96-well plate 24 h prior to incubation with liposomes. Prior to cell incubation, liposomal formulations were mixed with FFRPMI medium to a 10  $\mu$ M doxorubicin concentration and added immediately to cells.

Cells were incubated with the liposomal formulations for 2 h at 37 °C. Cells were then washed three times with fresh medium and reincubated for 72 h. The number of viable cells was determined with a formazan-based assay (CCK-8, Dojindo, Kumamoto, Japan) in a Synergy microplate reader (Bio-Tek, Winooski, VT).

For quantitative cytotoxicity evaluation, the liposomal doxorubicin concentration leading to 50% cell death (LC50) was determined. Cells were incubated with liposomes serially diluted from 51.2  $\mu$ M to 0.4  $\mu$ M doxorubicin concentrations in

1:2 dilutions. Cells were washed, reincubated and tested 72 h later for viability. LC50 values were determined from a best fit line of at least three points in the linear range of cell viability.

Cytotoxicity was quantified by determining the viability of treated cells relative to untreated controls (untreated control viability=100%). Cytotoxicity results are therefore reported as relative viability such that a higher relative viability corresponds to less toxicity.

#### 2.7. Construction and design of dual-ligand nanocarriers

Dual-ligand liposomes were constructed by a sequential process of DSPE-PEG3350-folate insertion followed by mAb225 coupling. The number of each type of ligand per liposome was determined by a ligand to phospholipid ratio, assuming 120,000 phospholipid molecules per liposome. As with the single-ligand liposomes, the number of each ligand determined represents an average of the bulk distribution. The viability profiles for single-ligand liposomes were utilized to design dual-ligand liposomes, applying several criteria. The number of ligands for control single-ligand liposomes was to be in the region of maximal efficacy. The number of each ligand in the dual-ligand case was to have viability similar to that of non-targeted liposomes (baseline toxicity), and the dual-ligand formulation was to have one-half the number of ligands of the single-ligand formulation that achieved maximal efficacy to ensure equal contribution of each ligand.

To obtain a dual-ligand formulation at or near the optimal number for each ligand, several dual-ligand formulations were tested. During this design process, the number of mAb225 per liposome was held constant because of the dramatic sensitivity of cell viability to the number of mAb225 targeting ligands in the low range ligands (see Results).

#### 2.8. Assaying targeting selectivity

For testing of targeting selectivity, an in vitro model system to simulate off-target cells bearing only one or none of the targeted receptors was made by blocking receptors on KB cells with excess free ligand (Fig. 1B). Off-target cells simulating those with FR but without EGFR were blocked with excess mAb225; off-target cells simulating those with EGFR but without FR were blocked with excess folic acid; and off-target cells simulating those without FR or EGFR were blocked with both excess folic acid and mAb225. For receptor blockade, 4 mM excess folic acid, 4  $\mu$ M excess mAb225 or both free ligands together were added to the culture medium for incubation with cells. Selectivity was determined by cytotoxicity with 10  $\mu$ M doxorubicin and LC50 values as described above.

#### 2.9. Imaging doxorubicin cellular localization by laser scanning confocal microscopy

Twenty-four hours before incubation with liposomes, KB cells were seeded at  $10^5$  cells per 18 mm glass coverslip. 10  $\mu$ M liposomal doxorubicin formulations were prepared as for cytotoxicity experiments.

Cells were incubated with doxorubicin liposomes for 120 min at 37 °C and 5% CO<sub>2</sub> in a humidified environment. Cells were then placed on ice, washed three times with ice cold PBS, fixed with 4% paraformaldehyde/1.5% methanol for 20 min [24] and counterstained with 10 μM DAPI. Coverslips were mounted to glass slides with FluoroMountG (Southern Biotech, Birmingham, AL).

Cells were imaged on a Zeiss Axiovert 2 microscope. Images were collected in Zeiss LSM510 software and processed in Adobe Photoshop CS.

### 3. Results

#### 3.1. Characterization of mAb225

1 g of purified mAb225 was obtained from the 40L bulk production. Antibody concentration was determined by the extinction coefficient compared to mouse IgG molecules. Immunohistochemistry with mAb225 showed positive signal in KB cells positive for the EGFR but not in 9L cells negative for the EGFR (data not shown).

#### 3.2. Characterization of DSPE-PEG3350-folate

DSPE-PEG3350-folate was characterized by thin layer chromatography, which indicated an absence of ninhydrin staining (reaction of amine groups), presence of lipid (cupric sulfate spray) and a fluorescent signal under a long-pass UV lamp (presence of folic acid) at an  $R_f$  value of 0.7 in an 80:20 chloroform/methanol mixture. Folate content of the conjugate was 93% of the theoretical maximum. <sup>1</sup>H-NMR confirmed the structure of final product, showing peaks for folic acid, PEG and DSPE similar to those described previously [16].

#### 3.3. Characterization of single-ligand nanocarriers

After extrusion liposome size was found to be 115–120 nm by dynamic light scattering. Insertion efficiencies of DSPE-PEG3350-folate were 70–75% in single- and dual-ligand liposomes. Coupling efficiencies were typically greater than 80% for mAb225 in single- and dual-ligand liposomes. Some of the resulting single-ligand folate and mAb225 liposomes were analyzed for particle size by dynamic light scattering. For the number of folate ligands utilized in most of these studies (200–400 folate ligands), an increase in diameter of 5–20 nm was observed compared to non-targeted liposomes. For the number of mAb225 ligands utilized in most of these studies (3–6 mAb225 ligands), no increase in diameter was observed compared to non-targeted liposomes (data not shown).

After folate conjugate insertion and mAb225 coupling, doxorubicin was loaded into liposomes. Loading efficiencies of 95–100% were routine.

#### 3.4. Cytotoxicity of single-ligand nanocarriers

Cytotoxicity as measured by viability as a function of ligand numbers is shown for each ligand in Fig. 2A and B. The abscissa

is the number of ligands per liposome while the ordinate is the viability relative to an untreated control (untreated control = 100%). As shown in Fig. 2A, 0–200 folate targeting ligands led

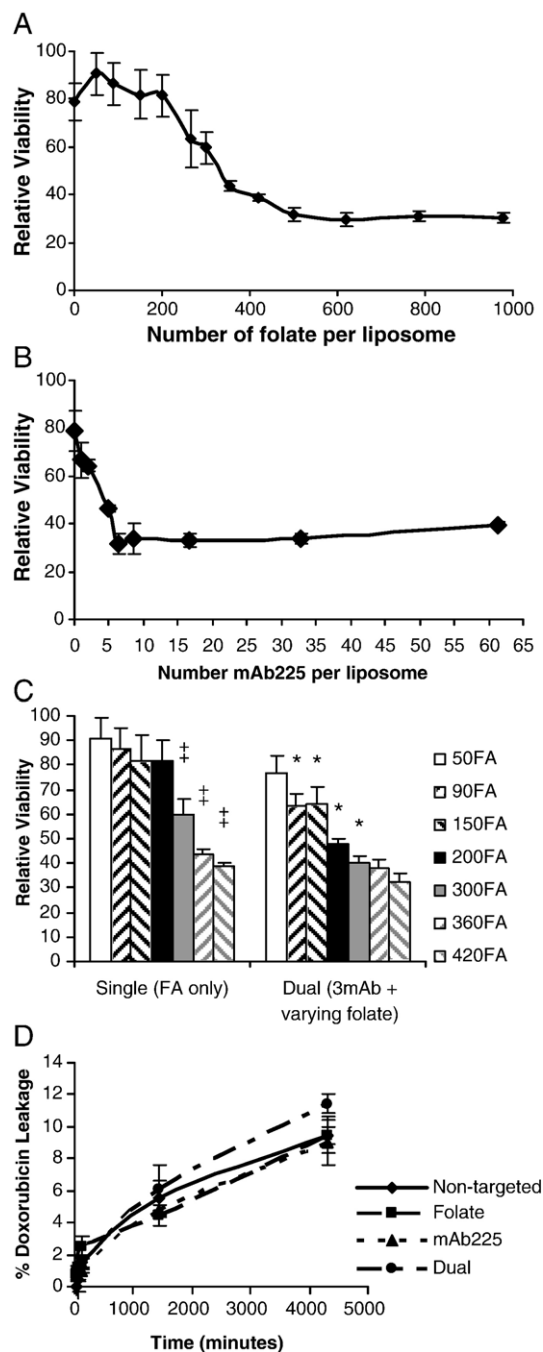


Fig. 2. Design and characterization of single- and dual-ligand liposomes. Cell viability relative to untreated control after exposure to liposomal formulations bearing (A) folate or (B) mAb225 ligands. (C) The optimal dual-ligand formulation was ensured by testing several formulations. One of these (200 folate+3 mAb225) was selected as the optimal formulation for further testing. (D) Doxorubicin leakage from non-targeted, folate-targeted, mAb225-targeted or dual-ligand liposomes.  $N=3$  or greater for all data points, error bars denote standard deviation. \* denotes  $P<0.05$  between single and dual-ligand formulations and ‡ denotes  $P<0.01$  between single-ligand and non-targeted formulations (baseline). Significance was determined by Tukey's pairwise comparison with ANOVA analysis.

to viability near baseline, where baseline is defined as the viability of KB cells when treated with non-targeted liposomes (0 targeting ligands). At greater than 200 folate ligands per liposome, reduced viability was observed and a maximal effect (minimal viability) was achieved at approximately 400 folate ligands per liposome with no increase in toxicity at higher ligand numbers.

As shown in Fig. 2B, a dramatic decrease in viability with relatively few numbers of mAb225 ligands was observed with a minimum viability at 6 ligands per liposome. At numbers of ligands greater than the point of minimum viability, an increase in viability was observed.

### 3.5. Construction and design of dual-ligand nanocarriers and assessing cytotoxicity

For single-ligand folate carriers, the cell viability showed a continuous variation with the number of ligands per carrier (Fig. 2A) from 90 to 420 ligands per liposome. In the dual-ligand carriers, we therefore varied the number of folates per carrier over this range. In contrast, for single-ligand mAb225 liposomes, the cell viability was very sensitive to the number of mAb225 per liposome, particularly from 0 to 6 mAb225 per liposome (Fig. 2B). At larger ligand numbers, the viability was less sensitive to the number of ligands. This small range was difficult to reliably probe using our fabrication technique. We therefore fixed the mAb225 numbers at 3 per liposome for testing dual-ligand constructs.

Fig. 2C shows the relative viability of KB cells treated with single-ligand folate formulations (left panels) or with the corresponding dual-ligand formulations bearing a fixed number of 3 mAb225 per liposome (right panels). Comparison of the left and right panels shows the effect of the addition of the second ligand (mAb225) to the folate liposomes. With 50–150 folate ligands per liposome, relative viability achieved with folic acid alone was near baseline with no significant difference between any of the single-ligand formulations. The dual-ligand formulations bearing 90 and 150 folate per liposome had significantly reduced viability compared to their single-ligand counterparts. However, addition of three monoclonal antibodies to these folate formulations did not achieve a reduction in viability to below the LC50. Single-ligand liposomes bearing 300 folate molecules did show a significant reduction in viability with the addition of 3 mAb225 ligands per liposome. However, the 300 folate single-ligand formulation had a reduction in viability significantly below the baseline, non-targeted amount. For the single-ligand formulations bearing 360 and 420 folates per liposome, the amount of cytotoxicity achieved with folic acid alone was near the maximal efficacy (viability was significantly below baseline). Therefore, addition of 3 mAb225 antibodies to the formulations did not lead to a significant additional reduction in viability.

In contrast to the formulations described above, with the formulation bearing 200 folate ligands per liposome a significant and dramatic decrease was observed upon the addition of the second targeting ligand (mAb225) in sub-optimal numbers. For 200 folate targeting ligands in the single-ligand formulation, a baseline effect was observed. With the addition of 3 mAb225 antibodies to the formulation, a significant decrease was ob-

served that led to viability near the LC50. Thus, this formulation met the criteria that the number of each ligand was to be (a) one half the number of the single-ligand formulation showing maximal efficacy and (b) a number that with minimal toxicity near baseline. Other formulations bearing a single ligand either did not achieve toxicity with or without a second ligand, or they achieved toxicity with only a single ligand. It was therefore selected as the best formulation for evaluation of targeting selectivity.

Leakage of doxorubicin from liposomal nanocarriers was determined to ensure that the presence of one or two types of ligands did not lead to increased leakage. The presence of folic acid, mAb225

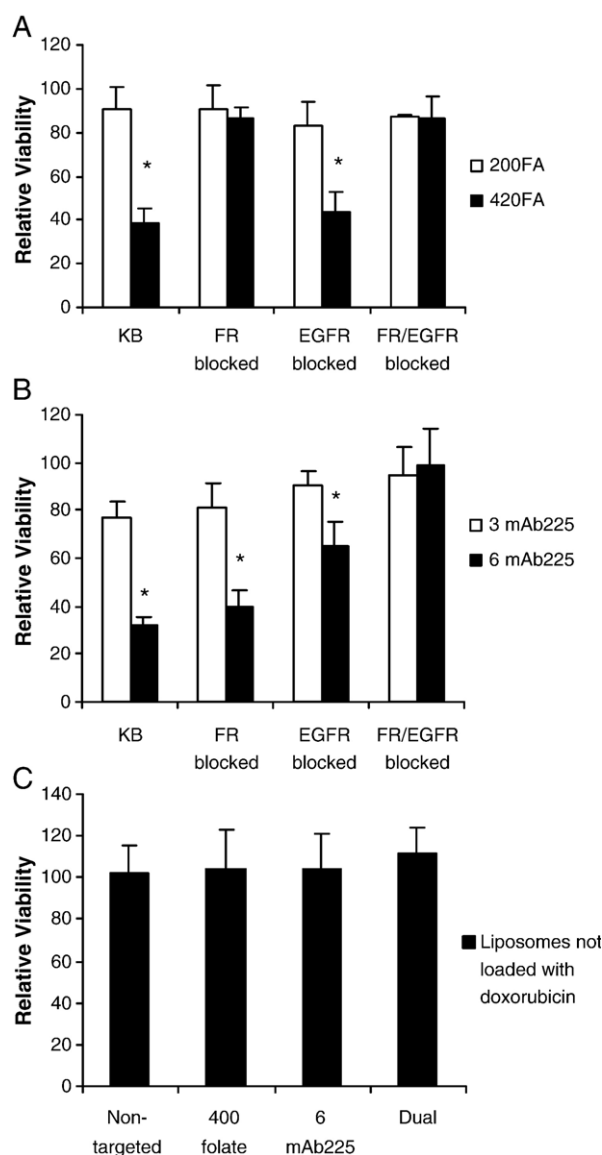


Fig. 3. Viability of target and off-target cells after exposure to single-ligand (A) folate and (B) mAb225 liposomes at a 10  $\mu$ M doxorubicin concentration. 200 folate and 3 mAb225 (sub-maximal efficacy) show baseline toxicity in all cell types. 400 folate and 6 mAb225 (maximal efficacy) led to near maximal efficacy in KB cells and also in at least one type of off-target control cell. (C) Liposomes not loaded with doxorubicin did not show toxic effects.  $n > 6$  for (A) and (B),  $n = 3$  for (C), error bars denote standard deviation. \* denotes  $P < 0.05$  between maximal efficacy and sub-maximal single-ligand formulations as determined by Tukey's pairwise comparison with ANOVA analysis.

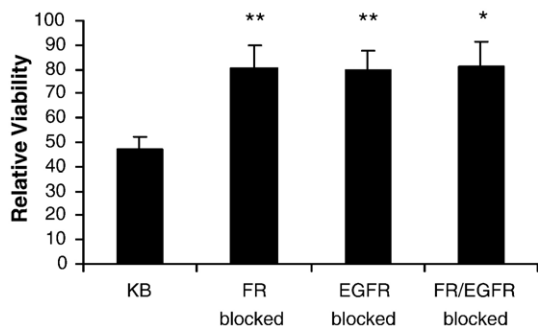


Fig. 4. Dual-ligand liposomes bearing the optimized number of each targeting ligand show near maximal efficacy only in the target cells (KB cells).  $n=6$  or greater for KB cells, EGFR blockade, and FR blockade,  $n=3$  for EGFR and FR dual blockade. \*\* denotes  $P<0.01$  between KB cells and indicated level, \* denotes  $P<0.05$  between KB cells and indicated level as determined by Student's *t*-test.

or both together on liposomes did not lead to a significantly increased amount of doxorubicin leakage relative to non-targeted liposomes at any time between 30 min and 3 days (Fig. 2D).

### 3.6. Testing selectivity of single- and dual-ligand nanocarriers

Fig. 3A and B show the viability of single-ligand nanocarriers in each of four cell conditions: KB cells, only the EGFR available (FR blocked), only the FR available (EGFR blocked) and neither receptor available (FR/EGFR blocked). Shown are the sub-maximal and maximal efficacy ligand numbers for each type of single-ligand carrier. Specifically, 200 folate ligands or 3 mAb225 per liposome were considered sub-maximal for single-ligand formulations as they did not show reduction in viability below that of non-targeted liposome baseline in any cell type (target or off-target), whereas ~400 folate ligands and 6 mAb225 per liposome were maximal as they achieved the maximal reduction in viability (Fig. 2A and B).

The single-ligand formulations leading to maximal efficacy (~400 folates or 6 mAb225 per liposome) are reflected in the low relative viabilities in target cells (KB) in Fig. 3A and B (black bars). However, these formulations also led to low relative viabilities in off-target cells when the receptor corresponding to that ligand was available, as indicated by reduced viability in EGFR blocked (FR available) with maximal efficacy folate liposomes (~400 folates) and reduced viability in FR blocked (EGFR available) with maximal efficacy mAb225 (6 mAb225) liposomes.

An additional control in which non-targeted, folate-targeted, mAb225-targeted or dual-ligand liposomes were not loaded with doxorubicin did not show a reduction in viability to untreated controls (100% viability), indicating that observed toxicities were due to doxorubicin (Fig. 3C).

Fig. 4 shows the viability of each of the target and off-target cells (where one or both receptors were blocked) tested with dual-ligand nanocarriers. In contrast to Fig. 3A and B, only the target cells (KB) bearing both targeted receptors showed reduced viability, demonstrating the significant targeting enhancement with dual-ligand liposomes. Cells bearing one or none of the targeted receptors exhibited only baseline viability.

To further 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

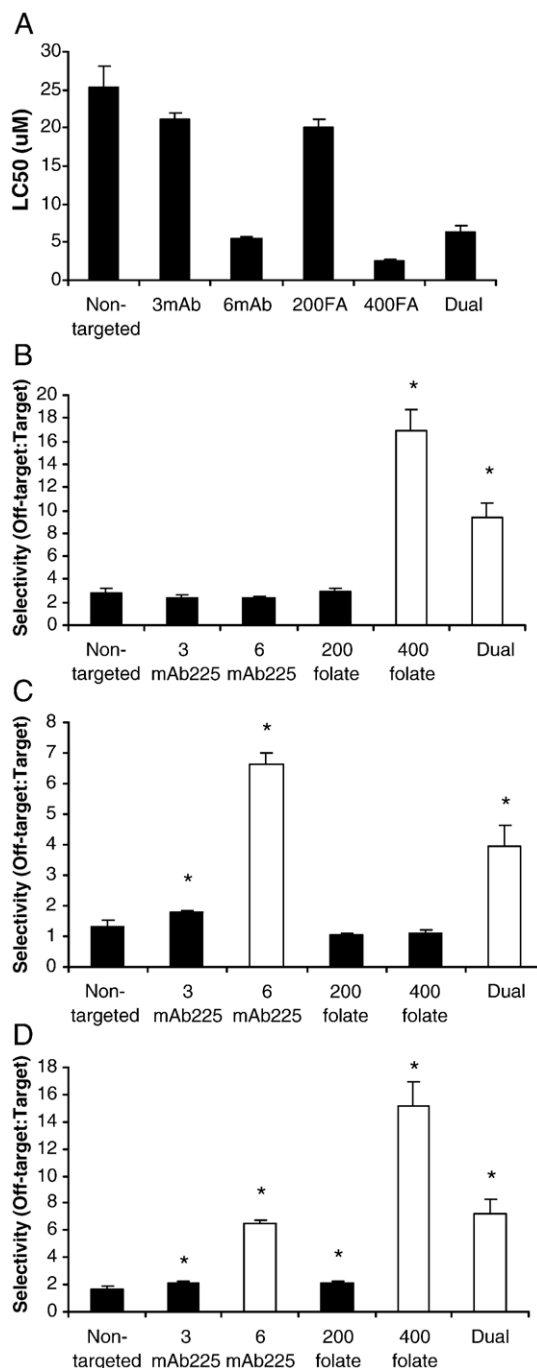


Fig. 5. (A) LC50 values for the various liposomal-doxorubicin formulations tested provide evidence for targeting selectivity enhancement with dual-ligand liposomes. Selectivity of the liposomal-doxorubicin formulations for (B) FR blockade of off-target cells (only EGFR available on off-target cells), (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.

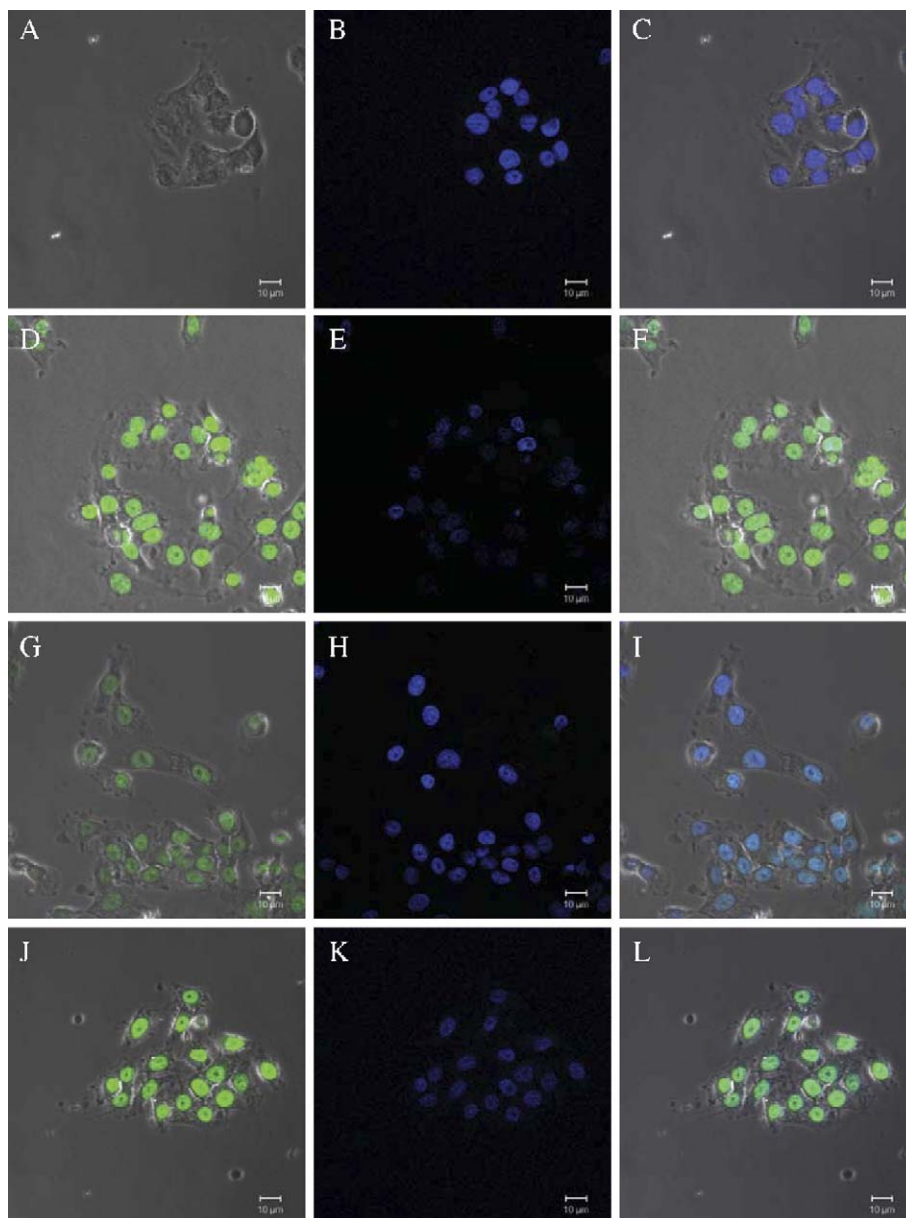


Fig. 6. (A), (D), (G) and (J) show a phase contrast image overlay with doxorubicin confocal slice image in KB cells. (B), (E), (H) and (K) show a DAPI confocal counterstain image for the same cells. (C), (F), (I) and (L) show the phase/DAPI overlay with DAPI. (A–C) show no doxorubicin uptake with non-targeted liposomes. Folate- (D–F), mAb225- (G–I) and dual-ligand- (J–L) targeted liposomes show nuclear localization. Scale bars denote 10  $\mu\text{m}$ .

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. 5A 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. 5B–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. 5B), 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. 5C), 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. 5D), 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.

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. 5B–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.

### 3.7. 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. 6 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. 6A–C). Folate-targeted liposomes (Fig. 6D–F), mAb225-targeted liposomes (Fig. 6G–I) and dual-ligand liposomes (Fig. 6J–L) all showed nuclear localization of doxorubicin after 2 h of treatment as indicated by co-localization of doxorubicin and DAPI.

## 4. Discussion

One challenge to the use of targeting ligands for directing nanocarriers to over-expressed tumor cell receptors (or other epitopes) is the ability to selectively deliver therapeutic agents to these cells while sparing off-target (healthy) cells. It is widely accepted that targeting ligands directing therapeutic agents (or, in the case of monoclonal antibodies, themselves acting as therapeutic agents) can be used to select between cells with vastly different receptor profiles for a given receptor [25–27]. However, in cases in which populations of off-target (healthy) cells express the target receptor, selecting between target and off-target cells becomes problematic.

This study describes a new approach to overcome non-selective ligand binding to off-target cells by exploiting the coincident over-expression of multiple receptors on targeted tumor cells. Dual-ligand nanocarriers were designed to deliver cytotoxic amounts of doxorubicin only to target cells in which both targeted receptors were available for binding.

Use of targeted nanocarriers for drug delivery has met with varied degrees of success in vivo [13,28,29]. This is likely due primarily to the two-step process involved in delivery of these agents: (1) extravasation followed by (2) receptor-mediated binding/internalization of the drug. Efforts to enhance extravasation [30] may ultimately allow more successful receptor targeting in step 2 of the process, although additional work is required to harness targeting methodologies. However, the in vivo barriers were removed from the system by use of an in vitro model in order to test the validity of the dual-ligand hypothesis. This

approach also removes problems of unknown in vivo receptor profiles and clearance issues, including opsonization of liposomes targeted with small molecules and antibodies, to focus on the utility of the targeting mechanism itself.

Reports utilizing two types of ligands in conjunction on targeted nanocarriers are rare, and none of these sought to modulate ligand numbers for selectivity enhancement. Zhang et al. described a two-step approach in which one ligand enhances blood–brain barrier permeability while a second ligand mediates tumor cell binding for antisense [31] and siRNA delivery [32], but the two receptors were targeted sequentially on different cells rather than simultaneously. Sapra and Allen utilized a dual targeting approach by simultaneously utilizing internalizing and noninternalizing antibodies against CD-19 and CD-20, respectively [33]. Although not bound to a single carrier, the combinatorial delivery of ligands directed against internalizing and noninternalizing antibodies led to an enhancement of drug uptake. The use of a dual-ligand approach to adenoviral targeting to EGFR and the  $\alpha\beta 5$  integrin has been explored [34], but an additive approach to ligand numbers was utilized and no effort was made to regulate the numbers of ligands to control off-target delivery. The use of two ligands on drug carriers has been demonstrated to confer microparticles with leukocyte rolling/adhesion properties [35] and to elicit improved induction of immune response for anti-tumor vaccinations [36].

However, the simultaneous use of dual targeting ligands directed at co-expressed tumor cell receptors and the development of techniques to optimize such a strategy for selectivity enhancement have not been described. Here, ligands (folic acid and mAb225) against two commonly targeted receptors (FR and EGFR) were used simultaneously to enhance selectivity. Different tether lengths for the two ligands (PEG3350 for folic acid and PEG2000 for mAb225) were used to account for differences in molecular weights of the ligands (441 Da for folic acid and 150,000 Da for mAb225). The total length of the folic acid plus PEG3350 is approximately 270 Å, while the length of mAb225 plus PEG2000 is approximately 268 Å when the tethers are fully extended, theoretically allowing nearly equal access of each ligand to their receptors.

The folic acid and mAb225 ligands were incorporated and conjugated, respectively, in successive order to allow characterization after each step. The DSPE-PEG3350-folate conjugate was incorporated by a post-insertion technique that allows for incorporation into the external leaflet of the bilayer in a fashion allowing us control over the number of targeting ligands present. While it is conceivable that the DSPE-PEG2000-maleimide in the liposomal formulation could distribute out of the bilayer and into the DSPE-PEG3350-folate micelles, this is unlikely as the DSPE-PEG2000-maleimide is in a more favorable energetic state while in the bilayer. The lipid tails would have to expose themselves to the aqueous phase in order to distribute into the DSPE-PEG3350-folate micelles. In contrast, the DSPE-PEG3350-folate is more likely to distribute to the liposomal phase because of the partition of the DSPE-PEG3350-folate between micelle and monomer states. When in the monomer state, it is favorable for the conjugate to enter the lipid bilayer. The stability

of DSPE-PEG2000-maleimide in the bilayer is further demonstrated by our ability to couple nearly 100% of the theoretical number of thiolated antibodies to liposomes, indicating the presence of the reactive maleimide group on the surface.

Liposomes were used as the nanocarrier system due to their long circulating time in vivo [37,38], amenability to modification with targeting ligands in a controlled fashion, and ability to encapsulate a variety of different therapeutic agents [39–41]. Doxorubicin was used as the drug to assess the dual-ligand hypothesis because it is widely used, has FDA approval in liposomal form (DOXIL), and can be tracked by fluorescence and toxicity. While a liposomal-doxorubicin system was utilized here, the dual-ligand approach should be applicable to other receptor-targeted carriers and therapeutic agents [42–44], albeit with further optimization requirements.

The objective of the dual-ligand approach was to utilize a number of each targeting ligand that, alone, would be insufficient to elicit toxicity but would elicit toxicity when utilized simultaneously. The number of each ligand to utilize was determined from toxicity profiles for each single-ligand liposome. For dual-ligand liposomes, one half the number of ligands achieving maximal efficacy for single-ligand liposomes was utilized to ensure equal contribution by both ligands.

For both single-ligand formulations, the change in viability as a function of ligand number is likely related to the amount of doxorubicin uptake. The trend observed for cytotoxicity as a function of ligand number corresponds to the pattern observed for the amount of doxorubicin uptake [16] (and unpublished results). The mechanism resulting in the sharp decrease and plateau for mAb225-targeted liposomes is unclear, but may be related to the bivalent nature of the mAb225 antibody. An optimal number of targeting ligands per liposome may exist such that upon the binding of one of the valent sites of the IgG, EGF receptors recruited to the location of the binding event may interact with the same IgG, enhancing the strength of the ligand–receptor bond. Use of additional targeting ligands may lead to interaction between different IgG molecules on the same liposome when EGF receptors are recruited to the area, thereby decreasing the overall affinity of the liposome–cell interaction. Although bivalency may play a role, others have observed a plateau in drug uptake with the Fab' fragments of the human chimerized version of the antibody, albeit over a more limited range of ligand numbers [45].

Cells treated with folate, mAb225 or dual-ligand liposomes without doxorubicin showed no reduction in viability, indicating that doxorubicin and not the ligands themselves were responsible for the cytotoxic effect (Fig. 3C).

A variety of dual-ligand formulations were constructed to rationally arrive at an 'optimal' formulation. For both single- and dual-ligand formulations, only average number of ligands per liposome was determined, as estimated from a ratio of the ligand concentration to lipid concentration (determined by DPH fluorescence) and assuming 120,000 lipid molecules per liposome. Thus, the resulting determination of the number of ligands per liposome is an average for the bulk formulation. It is likely that there is some distribution of each ligand around this mean value. The dual-ligand formulations are therefore likely

to be heterogeneous in the actual number of ligands per liposome. Obtaining formulations monodisperse in a single or dual-ligand targeting ligand (i.e., containing an exact number of ligands) are difficult and techniques to achieve this in liposomes, to the best of our knowledge, have not been reported. In one study (data not shown) the cytotoxicity was determined as a function of doxorubicin concentration for single-ligand formulations mixed together in sub-optimal numbers. The behavior of this system in terms of cytotoxicity and selectivity differed from the dual-ligand formulations, indicating that the dual-ligand liposomes did contain both targeting ligands. The presence of both ligands on the liposomes for in vivo applications may allow a more uniform distribution of the liposomes, and the use of sub-optimal ligand numbers in the dual-ligand case should help to minimize uptake in off-target cells in vivo. However, an in vivo comparison of co-single-ligand injections [33] and the dual-ligand formulations described here will help to further clarify the advantages of each approach.

The number of mAb225 was held constant due to the sensitivity to change in the number of this ligand, while the number of folate ligands was varied. The 200 folate formulation showed the greatest decrease in viability from the single to the dual-ligand formulations. Utilization of fewer folate ligands led to a significant, but not dramatic, decrease in viability while utilization of greater than 200 folate ligands led to a near-maximal efficacy even for single-ligand formulations. Thus, the selected formulation for folic acid and mAb225 is most likely to avoid off-target cell toxicity.

Use of single-ligand formulations led to a reduction in viability (increased toxicity) not only in target KB cells but also in off-target cells with the targeted receptor available. While this system is an in vitro model, the results are likely indicative of behavior that may be observed in vivo. For example, while a number of tumors have been reported to express the folate receptor, it has been shown folate receptor expression is also present in the choroid plexus, lung, thyroid and kidney [4] as well as in activated macrophages of the liver (Kupffer cells) [46]. Similarly, the EGFR is expressed on a variety of epithelial tissues in the body [47]. This indicates that when properly designed, the use of two (or more) targeting ligands in optimal numbers may reduce the uptake of drug in these cells by reducing the possible number of ligand–receptor interactions.

Unlike single-ligand targeting, dual-ligand liposomes reduced viability only in target cells bearing both targeted receptors while sparing off-target cells. Selectivity enhancements determined by LC50 ratios for single- and dual-ligand formulations showed that dual-ligand liposomes were capable of achieving a 10-fold enhancement relative to off-target cells without the folate receptor and a 4-fold enhancement relative to off-target cells without the EGFR. Although the single-ligand formulations achieving maximal efficacy (~400 folate and 6 mAb225) were capable of achieving similar cytotoxicity as the dual-ligand formulation, only the dual-ligand formulation was capable of selectivity enhancement relative to all off-target cells. These results in conjunction with the experiments at 10  $\mu$ M doxorubicin concentration indicate the potential utility of a

dual-ligand approach to enhance targeting selectivity of nano-carriers for tumor targeting.

Concentrations of liposome-encapsulated doxorubicin spanning two orders of magnitude were tested to determine LC50 values. For all formulations tested for LC50 values over the two orders of magnitude doxorubicin concentration difference as well as for any single-ligand formulation tested at 10  $\mu$ M over a range of targeting ligands, approximately 30% relative viability was observed. A multi-drug efflux pump could explain the observed behavior, but we have found that free doxorubicin can achieve 100% toxicity at a 10  $\mu$ M concentration (data not shown) in this cell line. Kinetic effects of doxorubicin transport or drug release are unlikely to explain differences in free drug compared to liposomally encapsulated drug as we have observed rapid trafficking of doxorubicin to cell nuclei with fixed cells in time course. A possible cause to this behavior that cannot be ruled out is that a sub-population of cells expressing insufficient numbers of receptors for targeting may be present.

The free ligands utilized for receptor blockade in the *in vitro* model system did not show toxic effects to the cells. Particularly, while anti-growth factor receptor antibodies have been shown to cause decreased cell proliferation [48,49], use of mAb225 was not observed to be detrimental to these cells in our study due to the short duration of exposure of the free antibody in high concentrations, as opposed to longer term exposure to the antibody. The lack of toxicity of the antibodies is indicated by similar viability between mAb225-blocked off-target cells and non-mAb225-blocked target cells.

A cellular localization study showed that the use of two different targeting ligands did not result in differences in the localization of doxorubicin. This indicates that, for these two ligands, use of different ligands with potentially different uptake pathways did not inhibit or prevent nuclear localization of doxorubicin.

Extension of the dual-ligand principle to cells with different receptor profiles for FR and EGFR or for different ligand–receptor pairs (e.g., transferrin/transferrin receptor or targets of high affinity phage-derived peptides) will likely require their own optimization processes. Additionally, the eventual use of more than two targeting ligands may be used to further enhance selectivity. However, optimization of targeted nanocarriers for given tumor cells with variant receptor profiles and nanocarriers with the corresponding ligands will likely prove to be a challenge. Further the response to a given number of each type of targeting ligand may differ *in vivo* due to clearance issues or different receptor expression patterns. Therefore, the ability to mathematically model the multi-valent ligand–receptor interactions of targeted nanocarriers may prove useful in determining optimal numbers of ligands for different target cells and targeting ligands [17].

In conclusion, we report here the development of a dual-ligand system to enhance the selectivity of targeted nanocarriers. 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.

## Acknowledgements

The authors thank Robert Mittler and Emily Burnham (Emory Vaccine Center Immunology Core Laboratory) for large-scale production of mAb225. This work was funded by the National Science Foundation, Bioengineering and Environmental Systems (BES) Award Number 0401627 (RVB).

## References

- [1] S.H. Torp, E. Helseth, A. Dalen, G. Unsgaard, Epidermal growth factor receptor expression in human gliomas, *Cancer Immunol. Immunother.* 33 (1) (1991) 61–64.
- [2] J. Santini, J.L. Formento, M. Francoual, G. Milano, M. Schneider, O. Dassonville, F. Demard, Characterization, quantification, and potential clinical value of the epidermal growth factor receptor in head and neck squamous cell carcinomas, *Head Neck* 13 (2) (1991) 132–139.
- [3] V. Rusch, J. Mendelsohn, E. Dmitrovsky, The epidermal growth factor receptor and its ligands as therapeutic targets in human tumors, *Cytokine Growth Factor Rev.* 7 (2) (1996) 133–141.
- [4] S.D. Weitman, R.H. Lark, L.R. Coney, D.W. Fort, V. Frasca, V.R. Zurawski Jr., B.A. Kamen, Distribution of the folate receptor GP38 in normal and malignant cell lines and tissues, *Cancer Res.* 52 (12) (1992) 3396–3401.
- [5] L. Recht, C.O. Torres, T.W. Smith, V. Raso, T.W. Griffin, Transferrin receptor in normal and neoplastic brain tissue: implications for brain-tumor immunotherapy, *J. Neurosurg.* 72 (6) (1990) 941–945.
- [6] B.J. Simpson, H.A. Phillips, A.M. Lessells, S.P. Langdon, W.R. Miller, c-erbB growth-factor-receptor proteins in ovarian tumours, *Int. J. Cancer* 64 (3) (1995) 202–206.
- [7] G. Blume, G. Cevc, M.D. Crommelin, I.A. Bakker-Woudenberg, C. Klufft, G. Storm, Specific targeting with poly(ethylene glycol)-modified liposomes: coupling of homing devices to the ends of the polymeric chains combines effective target binding with long circulation times, *Biochim. Biophys. Acta* 1149 (1) (1993) 180–184.
- [8] V.T. Kung, C.T. Redemann, Synthesis of carboxyacyl derivatives of phosphatidylethanolamine and use as an efficient method for conjugation of protein to liposomes, *Biochim. Biophys. Acta* 862 (2) (1986) 435–439.
- [9] A.N. Lukyanov, T.A. Elbayoumi, A.R. Chakilam, V.P. Torchilin, Tumor-targeted liposomes: doxorubicin-loaded long-circulating liposomes modified with anti-cancer antibody, *J. Control. Release* 100 (1) (2004) 135–144.
- [10] H.S. Yoo, T.G. Park, Folate receptor targeted biodegradable polymeric doxorubicin micelles, *J. Control. Release* 96 (2) (2004) 273–283.
- [11] Y. Xie, L. Ye, X. Zhang, W. Cui, J. Lou, T. Nagai, X. Hou, Transport of nerve growth factor encapsulated into liposomes across the blood–brain barrier: *In vitro* and *in vivo* studies, *J. Control. Release* 105 (1–2) (2005) 106–119.
- [12] D.A. Eavarone, X. Yu, R.V. Bellamkonda, Targeted drug delivery to C6 glioma by transferrin-coupled liposomes, *J. Biomed. Mater. Res.* 51 (1) (2000) 10–14.
- [13] C.P. Leamon, S.R. Cooper, G.E. Hardee, Folate-liposome-mediated antisense oligodeoxynucleotide targeting to cancer cells: evaluation *in vitro* and *in vivo*, *Bioconjug. Chem.* 14 (4) (2003) 738–747.
- [14] M. Bohl Kullberg, K. Mann, J.L. Owens, Improved drug delivery to cancer cells: a method using magnetoliposomes that target epidermal growth factor receptors, *Med. Hypotheses* 64 (3) (2005) 468–470.
- [15] T.M. Allen, E. Brandeis, C.B. Hansen, G.Y. Kao, S. Zalipsky, A new strategy for attachment of antibodies to sterically stabilized liposomes resulting in efficient targeting to cancer cells, *Biochim. Biophys. Acta* 1237 (2) (1995) 99–108.
- [16] J.M. Saul, A. Annapragada, J.V. Natarajan, R.V. Bellamkonda, Controlled targeting of liposomal doxorubicin via the folate receptor *in vitro*, *J. Control. Release* 92 (1–2) (2003) 49–67.

- [17] K.B. Ghaghada, J. Saul, J.V. Natarajan, R.V. Bellamkonda, A.V. Annapragada, Folate targeting of drug carriers: a mathematical model, *J. Control. Release* 104 (1) (2005) 113–128.
- [18] S. Zalipsky, E. Brandeis, M.S. Newman, M.C. Woodle, Long circulating, cationic liposomes containing amino-PEG-phosphatidylethanolamine, *FEBS Lett.* 353 (1) (1994) 71–74.
- [19] A. Gabizon, A.T. Horowitz, D. Goren, D. Tzemach, F. Mandelbaum-Shavit, M.M. Qazen, S. Zalipsky, Targeting folate receptor with folate linked to extremities of poly(ethylene glycol)-grafted liposomes: in vitro studies, *Bioconjug. Chem.* 10 (2) (1999) 289–298.
- [20] D.E. Lopes de Menezes, M.J. Kirchmeier, J.-F. Gange, L.M. Pilarski, T.M. Allen, Cellular trafficking and cytotoxicity of anti-cd19-targeted liposomal doxorubicin in B lymphoma cells, *J. Liposome Res.* 9 (2) (1999) 199–228.
- [21] E.M. Bolotin, R. Cohen, L.K. Bar, N. Emanuel, S. Ninio, D.D. Lasic, Y. Barenholz, Ammonium sulfate gradients for efficient and stable remote loading of amphipathic weak bases into liposomes and ligandoliposomes, *J. Liposome Res.* 4 (1) (1994) 455–479.
- [22] R.J. Lee, P.S. Low, Delivery of liposomes into cultured KB cells via folate receptor-mediated endocytosis, *J. Biol. Chem.* 269 (5) (1994) 3198–3204.
- [23] L. Qi, R.P. Singh, Y. Lu, R. Agarwal, G.S. Harrison, A. Franzusoff, L.M. Glode, Epidermal growth factor receptor mediates silibinin-induced cytotoxicity in a rat glioma cell line, *Cancer Biol. Ther.* 2 (5) (2003) 526–531.
- [24] D. Goren, A.T. Horowitz, D. Tzemach, M. Tarshish, S. Zalipsky, A. Gabizon, Nuclear delivery of doxorubicin via folate-targeted liposomes with bypass of multidrug-resistance efflux pump, *Clin. Cancer Res.* 6 (5) (2000) 1949–1957.
- [25] J. Sudimack, R.J. Lee, Targeted drug delivery via the folate receptor, *Adv. Drug Deliv. Rev.* 41 (2) (2000) 147–162.
- [26] H. Li, Z.M. Qian, Transferrin/transferrin receptor-mediated drug delivery, *Med. Res. Rev.* 22 (3) (2002) 225–250.
- [27] J. Baselga, The EGFR as a target for anticancer therapy-focus on cetuximab, *Eur. J. Cancer* 37 (Suppl 4) (2001) S16–S22.
- [28] O. Ishida, K. Maruyama, H. Tanahashi, M. Iwatsuru, K. Sasaki, M. Eriguchi, H. Yanagie, Liposomes bearing polyethyleneglycol-coupled transferrin with intracellular targeting property to the solid tumors in vivo, *Pharm. Res.* 18 (7) (2001) 1042–1048.
- [29] J. Huwyler, J. Yang, W.M. Pardridge, Receptor mediated delivery of daunomycin using immunoliposomes: pharmacokinetics and tissue distribution in the rat, *J. Pharmacol. Exp. Ther.* 282 (3) (1997) 1541–1546.
- [30] G. Kong, R.D. Braun, M.W. Dewhirst, Characterization of the effect of hyperthermia on nanoparticle extravasation from tumor vasculature, *Cancer Res.* 61 (7) (2001) 3027–3032.
- [31] Y. Zhang, C. Zhu, W.M. Pardridge, Antisense gene therapy of brain cancer with an artificial virus gene delivery system, *Mol. Ther.* 6 (1) (2002) 67–72.
- [32] Y. Zhang, Y.F. Zhang, J. Bryant, A. Charles, R.J. Boado, W.M. Pardridge, Intravenous RNA interference gene therapy targeting the human epidermal growth factor receptor prolongs survival in intracranial brain cancer, *Clin. Cancer Res.* 10 (11) (2004) 3667–3677.
- [33] P. Sapra, T.M. Allen, Improved outcome when B-cell lymphoma is treated with combinations of immunoliposomal anticancer drugs targeted to both the CD19 and CD20 epitopes, *Clin. Cancer Res.* 10 (7) (2004) 2530–2537.
- [34] J. Grill, V.W. Van Beusechem, P. Van Der Valk, C.M. Dirven, A. Leonhart, D.S. Pherai, H.J. Haisma, H.M. Pinedo, D.T. Curiel, W.R. Gerritsen, Combined targeting of adenoviruses to integrins and epidermal growth factor receptors increases gene transfer into primary glioma cells and spheroids, *Clin. Cancer Res.* 7 (3) (2001) 641–650.
- [35] A.O. Eniola, P.J. Willcox, D.A. Hammer, Interplay between rolling and firm adhesion elucidated with a cell-free system engineered with two distinct receptor–ligand pairs, *Biophys. J.* 85 (4) (2003) 2720–2731.
- [36] A. Roth, F. Rohrbach, R. Weth, B. Frisch, F. Schuber, W.S. Wels, Induction of effective and antigen-specific antitumor immunity by a liposomal ErbB2/HER2 peptide-based vaccination construct, *Br. J. Cancer* 92 (8) (2005) 1421–1429.
- [37] T.M. Allen, C. Hansen, F. Martin, C. Redemann, A. Yau-Young, Liposomes containing synthetic lipid derivatives of poly(ethylene glycol) show prolonged circulation half-lives in vivo, *Biochim. Biophys. Acta* 1066 (1) (1991) 29–36.
- [38] A.L. Klibanov, K. Maruyama, V.P. Torchilin, L. Huang, Amphipathic polyethyleneglycols effectively prolong the circulation time of liposomes, *FEBS Lett.* 268 (1) (1990) 235–237.
- [39] S. Wang, R.J. Lee, G. Cauchon, D.G. Gorenstein, P.S. Low, Delivery of antisense oligodeoxyribonucleotides against the human epidermal growth factor receptor into cultured KB cells with liposomes conjugated to folate via polyethylene glycol, *Proc. Natl. Acad. Sci. U. S. A.* 92 (8) (1995) 3318–3322.
- [40] E.B. Kullberg, M. Nestor, L. Gedda, Tumor-cell targeted epidermal growth factor liposomes loaded with boronated acridine: uptake and processing, *Pharm. Res.* 20 (2) (2003) 229–236.
- [41] H. Iinuma, K. Maruyama, K. Okinaga, K. Sasaki, T. Sekine, O. Ishida, N. Ogiwara, K. Johkura, Y. Yonemura, Intracellular targeting therapy of cisplatin-encapsulated transferrin-polyethylene glycol liposome on peritoneal dissemination of gastric cancer, *Int. J. Cancer* 99 (1) (2002) 130–137.
- [42] S.J. Chiu, N.T. Ueno, R.J. Lee, Tumor-targeted gene delivery via anti-HER2 antibody (trastuzumab, Herceptin) conjugated polyethylenimine, *J. Control. Release* 97 (2) (2004) 357–369.
- [43] X. Shuai, H. Ai, N. Nasongkla, S. Kim, J. Gao, Micellar carriers based on block copolymers of poly(epsilon-caprolactone) and poly(ethylene glycol) for doxorubicin delivery, *J. Control. Release* 98 (3) (2004) 415–426.
- [44] H.F. Liang, T.F. Yang, C.T. Huang, M.C. Chen, H.W. Sung, Preparation of nanoparticles composed of poly(gamma-glutamic acid)-poly(lactide) block copolymers and evaluation of their uptake by HepG2 cells, *J. Control. Release* 105 (3) (2005) 213–225.
- [45] C. Mamot, D.C. Drummond, U. Greiser, K. Hong, D.B. Kirpotin, J.D. Marks, J.W. Park, Epidermal growth factor receptor (EGFR)-targeted immunoliposomes mediate specific and efficient drug delivery to EGFR- and EGFRvIII-overexpressing tumor cells, *Cancer Res.* 63 (12) (2003) 3154–3161.
- [46] M.J. Turk, D.J. Waters, P.S. Low, Folate-conjugated liposomes preferentially target macrophages associated with ovarian carcinoma, *Cancer Lett.* 213 (2) (2004) 165–172.
- [47] D.S. Salomon, R. Brandt, F. Ciardiello, N. Normanno, Epidermal growth factor-related peptides and their receptors in human malignancies, *Crit. Rev. Oncol.Hematol.* 19 (3) (1995) 183–232.
- [48] J.D. Sato, T. Kawamoto, A.D. Le, J. Mendelsohn, J. Polikoff, G.H. Sato, Biological effects in vitro of monoclonal antibodies to human epidermal growth factor receptors, *Mol. Biol. Med.* 1 (5) (1983) 511–529.
- [49] J.A. Drebin, V.C. Link, M.I. Greene, Monoclonal antibodies specific for the neu oncogene product directly mediate anti-tumor effects in vivo, *Oncogene* 2 (4) (1988) 387–394.