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# Controlled targeting of liposomal doxorubicin via the folate receptor in vitro

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**Abstract**

Differential expression of folate receptor has been exploited to target liposomes to tumors. Astrogliomas express low folate receptor levels and are typically surrounded by normal cells expressing little or no folate receptors. While targeting cells with high over-expression of folate receptor (KB and HeLa) has been demonstrated, it is unclear whether targeting tumors expressing low levels of folate receptor is possible. In this study, it was demonstrated that optimizing the number of targeting ligands (folic acid) enables differential liposomal doxorubicin uptake in C6 glioma while sparing healthy cortical cells. By micellization of folate conjugates and their controlled insertion into pre-formed liposomes, tight control over the number of targeting ligands per liposome was demonstrated. Doxorubicin uptake in KB and C6 cells was dependent on the number of targeting ligands, while cortical cells showed increasing non-specific uptake with ligand number. Co-culture of C6 glioma with cortical cells confirmed preferential uptake in C6 glioma relative to cortical cells. A cell kill experiment showed that folate-targeted liposomal doxorubicin is cytotoxic and slows proliferation of KB and C6 cells with minimal effect on cortical cells. Therefore modulation of targeting ligand number enables significant differential uptake of doxorubicin in cells with low levels of folate receptor.

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## 1. Introduction

Targeting the folate receptor has shown considerable promise in mediating uptake of a variety of drugs [1–4], gene therapy products [5,6], and radiopharmaceuticals [7] when folic acid is conjugated to the drug or delivery vehicle. When coupled to the terminal end of poly(ethylene glycol) (PEG) chains of liposomes, folic acid and other targeting molecules have the flexibility to interact with their corresponding cell surface receptor [8] to mediate liposomal uptake via endocytosis [2]. In vitro experi-

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ments using folate targeting of the anti-cancer drug doxorubicin (DOX) have shown that KB and HeLa cells, which vastly over-express the folate receptor, show significant uptake of the drug while WI-38 fibroblast cells which do not express the folate receptor take up minimal amounts of the drug [1].

Such experiments with DOX and other agents have shown that significant differentiation in uptake can be obtained via folate targeted liposomes between cells with vast over-expression (e.g., KB cells, ovarian carcinomas) and those with no expression of the folate receptor [1]. In this study it was demonstrated that tumor systems in which low levels of folate receptor are expressed also provide a potential targeting site. One case in which tumor cells express low levels of the folate receptor in the presence of surrounding healthy tissue expressing no receptor is that of brain tumors [9]. A particular model of interest in our laboratory is the C6 rat glioma model. As shown in Table 1, it was found that although C6 glioma cells show limited expression of the folate receptor, with approximately 30-fold lower expression than KB cells (a human nasopharyngeal cell line), the level of expression is significantly higher than primary brain tissue analyzed from E9 chick cortical cells (cortical cells). In this study the feasibility of targeting these glioma cells via folate receptor while at the same time minimizing non-specific uptake in normal/healthy cells was investigated. In targeting cells with low levels of receptor expression in the presence of cells bearing no receptors, optimizing the number of targeting ligands becomes critically important. If too few targeting ligands are utilized, sufficient binding between the targeting vehicle and the targeted cells will not occur. If too many targeting ligands are utilized, non-specific binding at non-targeted cells may occur, or recognition and uptake by the reticulo-endothelial system (RES) may become problematic in vivo [10].

In this study, a modification of the previously utilized so-called ‘post-insertion’ technique [11–14] was used in order to create a variety of liposomal formulations bearing varying numbers of folate ligands from a single batch of liposomes. The number of ligands coupled to the liposomal surface was varied over a range from 30 to 1130 per liposome (0.02–0.75% of the total lipid formulation). By using liposomes bearing controlled numbers of targeting ligands, the DOX uptake as a function of the number of targeting ligands was determined in the two cell lines (human KB oral carcinoma cells and rat C6 glioma cells) and the primary cultures (cortical cells) to establish the differential drug uptake. It was found that KB cells take up significant amounts of DOX relative to the C6 cells or relative to normal cortical tissue taken from E9 chicks. Importantly, however, it was found that significant differentiation could be obtained between the C6 cells and the E9 chick cortical tissues by optimizing the number of targeting ligands per liposome. This specificity of folate targeting was maintained for folate receptor-positive cells when cells were co-cultured. Additionally, cell death in KB, C6, and healthy cortical cells was qualitatively evaluated, and it was demonstrated that with a single 2-h incubation of folic acid-targeted liposomal DOX increasing cell death occurs in KB and C6 glioma with little evidence of cell death in cortical cells.

## 2. Materials and methods

### 2.1. Materials

1,2-Distearoyl-*sn*-glycerophosphocholine (DSPC), 1,2-dipalmitoyl-*sn*-glycerophosphoethanolamine-poly(ethylene glycol)2000-amine (DPPE-PEG-amine), and cholesterol were obtained from Avanti Polar Lipids (Birmingham, AL). Radiolabeled ($^3$H) folic acid was obtained from American Radiolabeled Chemicals (St. Louis, MO). C6 and KB cell lines were obtained from American Type Culture Collection (Manassas, VA). Doxorubicin (DOX) was obtained from Ben Venue Laboratories (Bedford, OH). Folic acid and dicyclohexylcarbodiimide (DCC) were obtained from Sigma (St. Louis, MO). Dimethylsulfoxide (DMSO), pyridine, chloride, ammonium sulfate, and sodium dodecyl sulfate (SDS) were ACS grade or higher and were obtained from Fisher Scientific (Pittsburg, PA). A 300 000 molecular weight cut-off (MWCO) dialysis tubing was obtained from Spectra/Por (Dominguez, CA). Deuterated DMSO-d6 solvent for $^1$H NMR was obtained from Norell (Landisville, NJ). Thin-layer chromatography plates were obtained from Selecto Scientific (Suwanee, GA). Folate-free RPMI 1640 cell culture

medium and phosphate-buffered saline (PBS) were obtained from Gibco (Carlsbad, CA). Dulbecco's modification of Eagle's medium (DMEM), fetal bovine serum (FBS), and penicillin–streptomycin (pen–strep) were obtained from Cellgro (Herndon, VA). Silicone barriers for co-culture experiments were made from Sylgard 184 silicone elastomer kit, a product of Dow Corning.

### 2.2. Cell culture and isolation

C6 and KB cell lines were maintained in RPMI 1640 folate-free medium supplemented with 10% FBS and 1% pen–strep in T-75 flasks. Cells were passaged by trypsinization followed by two washings with RPMI 1640 folate-free medium.

Terminally differentiated primary cortical neurons were obtained from day 9 (E9) chick embryos. The two frontal lobes of the brain were removed, minced, and dissociated with 0.25% trypsin. The cells were then plated into collagen-coated 35-mm six-well plates.

Cortical cells were cultured on collagen coated wells in order for these cells to attach, spread, and more accurately reflect their in vivo morphology. Additionally, these cells were cultured for longer amounts of time for radiolabeling and DOX uptake experiments because observation suggested that the additional time was required to allow the cells to fully attach and spread cellular processes characteristic of these neural cells. Collagen coating and the additional culture time were not required for KB and C6 cells.

### 2.3. Folate receptor count by radio-labeling assay

Three cell types (KB, C6, and cortical cells) were screened for their cell surface folate content in order to characterize the receptor profiles of the cells. KB and C6 cells were seeded at $5\times10^5$ cells per well of 35-mm six-well plates and were placed in a cell culture incubator at 95% humidity and 5% $CO_2$ for 24 h. Cortical cells were cultured for 3 days following dissociation as described above. The number of folate receptors on the cell surfaces of each cell type was determined by using a [$^3$H]folic acid radiolabeling assay following methods similar to those published by others [15,16]. Briefly, cells were placed on ice for 3 min. They were then washed once with Hepes-buffered saline (HBSS) and twice with acidic saline (pH 3.5) for 1 min. After a final wash with HBSS, the cells were incubated for 1 h on ice with 3 pmol of [$^3$H]folic acid. Co-incubation with an excess (1 μM) free folic acid served as a negative control.

### 2.4. Liposome assembly/formation

Liposomes were selected as the drug targeting vehicles for the folate targeting strategy due to (i) previous demonstration of targetability, (ii) ease of construction, (iii) compatibility with the 'post-insertion' technique to allow tight control over the number of targeting ligands per liposome, and (iv) ability to achieve high drug:delivery ratio for the targeting drug (DOX). Liposomes were constructed by methods similar to those described elsewhere [17,18]. Briefly, a 2:1 molar ratio of DSPC to cholesterol was dissolved in ethanol at 60 °C. The ethanol solution was then hydrated with a 200 mM ammonium sulfate buffer, which also facilitates DOX loading. Liposomes were extruded on a 10-ml Lipex Thermoline extruder (Northern Lipids, Vancouver, British Columbia, Canada) with five passes through a 0.2-μm Nuclepore membrane (Whatman, Newton, MA) and 10 passes through a 0.1-μm membrane. Following extrusion, the liposomal diameter was determined with a dynamic light scattering (DLS) apparatus. The DLS laser was a JDS uniphase μ-green laser at 532 nm wavelength. The DLS detector was a Brookhaven Instruments TFL detector (Brookhaven Instruments Corporation, Holtsville, NY) and data were analyzed with Brookhaven Instruments Light Scattering Software Version 3.16 (Brookhaven Instruments).

### 2.5. Synthesis and micellization of DPPE-PEG$_{2000}$-folate

A lipid-PEG-folate conjugate was synthesized that could later be incorporated into liposomes for targeting encapsulated DOX to cells via the folate receptor. The conjugate, 1,2-dipalmitoyl-*sn*-glycerophosphoethanolamine - poly(ethylene glycol)$_{2000}$-folate (DPPE-PEG$_{2000}$-folate), was constructed by methods similar to those described previously [3].

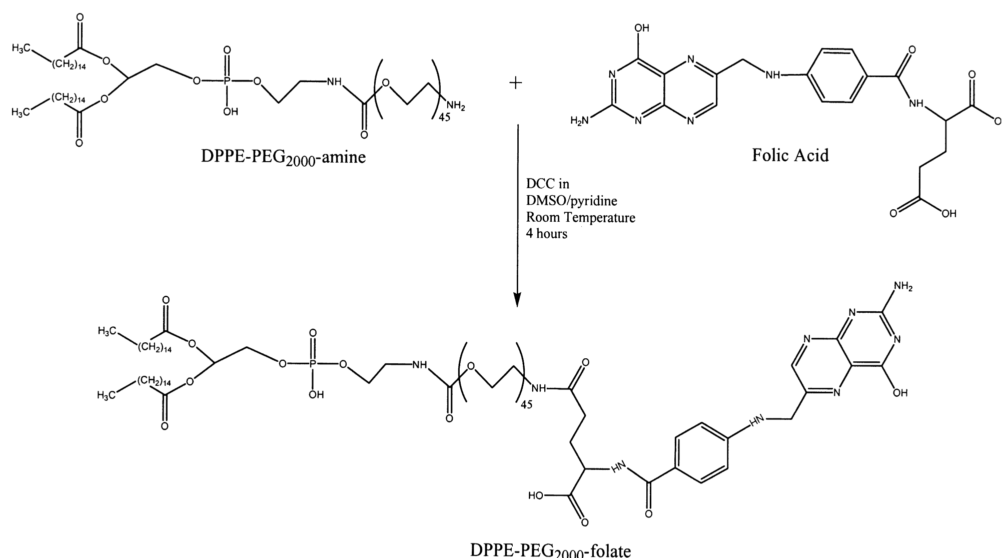



Fig. 1. Schematic of synthesis of DPPE-PEG$_{2000}$-Folate. The compound was synthesized by dicyclohexylcarbodiimide (DCC) chemistry linking the amine group of DPPE-PEG-amine and the γ-carboxyl of folic acid. The synthesis was confirmed by $^{1}$H NMR, mass spectroscopy, and thin-layer chromatography.

Fig. 1 summarizes the reaction scheme and product. Briefly, 16.7 mg of folate were dissolved in 667 μl of DMSO. A total of 66.7 mg DPPE-PEG-amine and 333 μl of pyridine was added to the reaction solution along with 21.7 mg of DCC. The reaction was allowed to proceed for 4 h at room temperature. The pyridine was removed by rotary evaporation and 16.7 ml of water was then added to the solution. The product was dialyzed with 300 000 MWCO dialysis tubing twice against 2 l of 50 mM sodium chloride and three times against 2 l of water. The solution was then lyophilized, yielding 66.6 mg (92% yield). The final product was analyzed by thin-layer chromatography, $^{1}$H NMR, and mass spectroscopy.

For thin-layer chromatography a 1.48 N ammonium hydroxide mobile phase was prepared as described elsewhere [19]. Samples were diluted in 0.3 N ammonium hydroxide and spotted in lanes on two separate plates. DPPE-PEG$_{2000}$-amine starting material and folic acid were run as controls along with product (DPPE-PEG$_{2000}$-folate). The plate was initially resolved by UV light. The presence of phospholipids was determined by resolving the plate with a cupric sulfate spray. The presence or absence of free amine was resolved separately by ninhydrin spray.

$^{1}$H NMR was performed on a Varian Inova 600 MHz NMR in the Case Western Reserve University Chemistry Department. DMSO-d6 deuterated solvent was used for sample analysis. Mass spectroscopy was performed at the Cleveland Center for Mass Spectroscopy on a Quatro II triple quadrupole mass spectrometer (Micromass, Manchester, UK) with ionization performed by electrospray ionization in the positive mode.

### *2.6. DPPE-PEG$_{2000}$-folate insertions into pre-formed liposomes*

In order to control the number of folate targeting ligands per liposome by 'post-insertion' of the conjugate into pre-formed liposomes [12], a method was developed to micellize the DPPE-PEG$_{2000}$-folate conjugate for insertion into the liposomes. Micelles were formed by dissolving 2 mg DPPE-PEG$_{2000}$-FA in 637 μl of DMSO. The solution was then hydrated with 5.7 ml of water, giving a solution with 10% DMSO. The 100 μM micelle suspension was then dialyzed three times in 300 000 MWCO dialysis tubing against 2 l of water to remove DMSO. Following dialysis, micelles were lysed in 10% SDS and the UV absorbance was measured at 285 nm

wavelength on a UV-visible spectrometer (Shimadzu Scientific Instruments Model 1601, Columbia, MD). The total amount of folate and the number of folate molecules per liposome were determined by comparison of the $UV_{285}$ reading to a standard curve of folic acid and the known lipid concentration.

Liposomes bearing between 30 and 1500 targeting ligands, which is between 0.02 and 1% of the total lipid formulation, were fabricated. Liposomes with no targeting ligands (conventional liposomes) were also fabricated. In order to construct folate liposomes, aliquots of a single batch of 15 mm liposomes (10 mM phospholipids, 5 mM cholesterol) were placed in separate vials. The number of liposomes present was determined based on the number of phospholipid molecules necessary to form a liposome of the given diameter (130 nm as determined by dynamic light scattering) assuming a phospholipids head group diameter of 0.65 $nm^2$. The number of folate ligands required for a given formulation (between 30 and 1500) was added to the liposomal suspension as aliquots of micellized DPPE-$PEG_{2000}$-FA at a lipid concentration of 100 μM, similar to previously published methods [11]. The liposomes and DPPE-$PEG_{2000}$-folate were then heated at 60 °C for 1 h. Following heating, the liposomes were cooled on ice. The suspension was then dialyzed overnight to remove any leaked ammonium sulfate and unincorporated DPPE-$PEG_{2000}$-folate from the external volume of the liposome suspension. Following dialysis, the folate content of the liposomes was determined by lysing the liposomes with 10% SDS and measuring the UV absorbance at 285 nm.

### *2.7. Active loading of DOX to folate-liposomes*

The drug DOX was selected for determining the targeting efficiency of folate liposomes because (i) it can be efficiently loaded into liposomes via a pH gradient, (ii) it is easily quantifiable due to its fluorescence properties, and (iii) an FDA approved non-targeted liposomal-DOX formulation (DOXIL®) is currently used clinically. Folate-liposomes were actively loaded with DOX by an ammonium sulfate gradient, as described previously [18]. Briefly, folate-liposomes and 2 mg/ml DOX were mixed at a ratio of 0.1 mg DOX per 1 mg of DSPC in the folate liposomes. The liposome/DOX suspension was heated at 60 °C for 1 h. The liposomes were then cooled immediately on ice and dialyzed twice in 300 000 MWCO membrane against 0.1 M sodium chloride to remove unencapsulated DOX. The final DOX concentration after dialysis was determined by lysis of the liposomes with 5% Triton X-100 and measurement of the UV absorbance at 480 nm [20]. Cell culture experiments were then performed immediately following dialysis of non-encapsulated DOX.

### *2.8. Cellular DOX uptake experiments*

The targeting efficiency as a function of ligand number was determined by in vitro incubation of cells with DOX-loaded folate-liposome formulations. After 40 h (C6 and KB) or 88 h (cortical neurons) in culture, the cultured cells were utilized for DOX uptake experiments by methods similar to those described elsewhere [1,21]. Briefly, after removing the culture medium, cells were washed once with PBS. Folate-free RPMI 1640 (1 ml) was then added to each well. DOX (100 μg) in either free form (free DOX) or liposomally encapsulated form was added to each well. Differences in liposomal DOX concentrations and with the concentration of free folic acid were corrected by diluting the medium with PBS to assure that DOX uptake effects were not due to concentration differences. Cells were then incubated for 2 h with the DOX mixture at 37 °C in a 5% $CO_2$, high humidity environment. Following incubation, cells were placed on ice, washed three times with 1 ml of ice-cold PBS containing calcium and magnesium to remove extracellular DOX. Cells were then lysed with 2 ml of 5% Triton X-100. The fluorescence intensity of the lysed cell solution was then measured on a fluorescence spectrometer (Perkin-Elmer LS55 Luminescence Spectrometer, Shelton, CT) at an excitation/emission of 475/580 nm.

Competitive binding experiments were performed to determine if folate ligands specifically mediated the cellular uptake of liposomal-DOX. In these experiments, 100 μg of DOX in folate-targeted liposomes bearing either 300 or 1130 targeting ligands were co-incubated with an excess of free folic acid at a 1 mM concentration. This method is designed to saturate folate receptors to prevent receptor-specific binding by folate liposomes, there-

by distinguishing between receptor-mediated and non-specific DOX uptake. An amout of 1 mM free folic acid is a 1000-fold excess over the number of folate ligands present due to the 300 folate ligands per liposome level and a 200-fold excess over the number of folate ligands present due to the 1130 folate ligands per liposome.

## 2.9. DOX uptake in co-culture

The specificity of the folate-targeted liposomes for cells expressing the folate receptor was determined by performing cellular co-culture experiments in which two cell types were co-cultured in a single 35-mm diameter well sharing the same culture medium and folate-liposomal DOX. Two types of cells (KB, C6, or cortical cells) were cultured in the same well of a 35-mm six-well plate.

In order to quantify DOX uptake, it was necessary to physically separate the two cell types to analyze DOX content separately on a fluorescence spectrometer. Therefore, a thin barrier of silicone was used to split 35-mm diameter wells of a six-well plate into two sections (see Fig. 2). This allowed cells to be cultured together without cross-contamination and to share the culture medium during DOX uptake experiments. Half the number of cells used in the single culture situation were used in order to obtain roughly the same cell density as used in the single culture.

Initially, half wells were collagen-coated for culturing cortical cells while the other half was untreated for culturing with C6 or KB cells. Cortical cells were then dissociated in RPMI 1640 folate-free medium as described earlier. These cells were cultured at a concentration of 500 000 cells in 0.4 ml of medium. The half wells of cortical cells were then cultured for 2 days alone in the half well. After 2 days, KB or C6 cells (at a concentration of 250 000 cells in 400 μl) were then added to the other half of the well and the co-cultured cells were incubated together for 40 h in folate-free medium. For KB/C6 co-cultures, no collagen treatment was utilized on either half of the well and cells were seeded in half wells at the same time.

Following the 40 h of co-incubation, cells were carefully washed with 1 ml of PBS followed by the addition of cell culture medium (1 ml). A total of 100 μg of DOX in liposomal form was then added and cells were incubated for 2 h with the DOX; sharing the same culture medium. Based on the results of the single cell type experiments, only the liposome formulation with 300 folate targeting ligands per liposome was used (see Section 4). Cells were then washed three times with ice-cold PBS and lysed with 5% Triton X-100, and removed from the wells according the cell types. The cell lysates were then analyzed for DOX content.

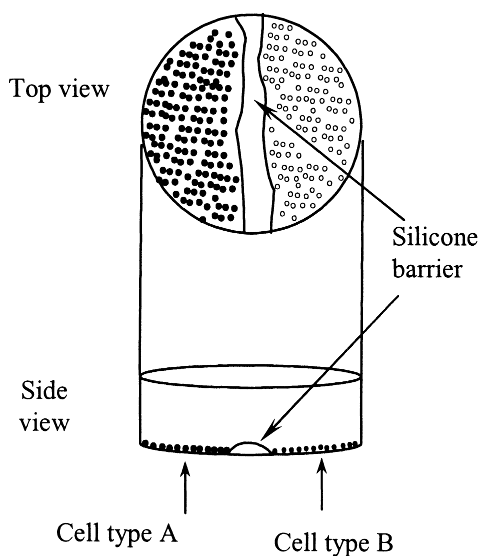



Fig. 2. Schematic of co-culture system. A top and side view of a 35-mm diameter well from a six-well plate used for the co-culture system is shown. Each well was split in half by a silicone barrier, and the plate was gas sterilized. Cells were added to each half of the well in 400 μl of medium, which came to a height just below the silicone barrier. After cell attachment, culture medium containing liposomal DOX in conventional or folate liposomes was added to the well. Two ml of medium covered the cells to a height above the level of the silicone barrier, allowing cells to share the same doxorubicin-containing culture medium over a 2 h incubation. The two cell types could then be lysed and removed from the culture plates separately for cellular DOX uptake.

## 2.10. Cell death due to DOX uptake

For clinical relevance, in addition to obtaining low non-specific uptake in healthy cells, sufficient uptake must occur in tumor cells to cause cell death. Therefore, KB, C6, and cortical cells were seeded and incubated with either conventional liposomes or

folate-targeted liposomes bearing 300 targeting ligands per liposome for 2 h. KB and C6 cells were seeded at a density of 50 000 cells per well of a 35-mm diameter plate. Cortical cells were seeded at a density of 500 000 cells per well because at lower cell densities the cells did not attach and spread processes. Despite the differences in cell number, at the start of the experiment cellular densities of all cell types were similar due to differences in cell size, morphology, and rate of proliferation (see Fig. 8A). After 40 h (KB and C6 cells) or 96 h (cortical cells) in culture, cells were incubated for 2 h under one of three conditions: (i) no treatment (culture medium only; control), (ii) 10 μM DOX (11.6 μg per well) in conventional liposomes (no folic acid), or (iii) 10 μM DOX (11.6 μg/well) in folate liposomes. Following the 2 h incubation, the DOX medium was removed, cells were washed carefully three times with fresh medium, 1 ml of fresh culture medium was added to the wells, and the cells were returned to the incubator. Cell proliferation and death were then observed by light microscopy over the next 6 days (144 h).

## 3. Results

### 3.1. Cell surface folate receptor determination by [$^3$H]folic acid radiolabel assay

KB cells were found to have approximately 280 000 cell surface folate receptors per cell, C6 glioma were found to have 10 000 cell surface folate receptors per cell, and terminally differentiated E9 cortical cells had no detectable folate receptors on their cell surface (see Table 1).

Table 1
Summary of cell surface folate receptor counts for each cell line as determined by $^3$H-folic acid radio-labeling assays as described in Section 2

| Cell type | Number of folate receptors per cell as determined by $^3$H-folic acid |
|---|---|
| KB cells | 279 000 ± 12 000 |
| C6 glioma cells | 9700 ± 270 |
| E9 chick cortical cells | None detectable |

### 3.2. Liposome characterization

The average diameter of extruded liposomes was 130 nm, as determined by dynamic light scattering. Liposomes were dialyzed in a 300 000 MWCO dialysis membrane once against 200 mM ammonium sulfate and then step-wise against decreasing sodium chloride concentrations to make the liposomes isotonic with cells and to establish an ammonium sulfate gradient for DOX loading [18].

### 3.3. DPPE-PEG$_{2000}$-folate synthesis and micelle formation analysis

A DPPE-PEG$_{2000}$-folate conjugate was constructed and micellized for later insertion into the pre-formed liposomes at well defined numbers of targeting ligands (see Table 2). The conjugate was made by dicyclohexylcarbodiimide chemistry in which the γ-carboxyl of folic acid was reacted with an amine-terminated poly(ethlylene glycol) lipid (DPPE-PEG$_{2000}$-amine) [3]. The final product is shown in Fig. 1. Final product yield was 92% (molar), as determined by mass and UV analysis of folate content. The product was analyzed by thin-layer chromatography (TLC), $^1$H NMR, and mass spectroscopy.

Thin-layer chromatography was performed on the product by using a mobile phase (1.48 N ammonium hydroxide) that would allow folic acid to move from the origin. The plate showed $R_f$ values of 0.49 for product and 0.31 for folic acid under UV (DPPE-PEG-amine did not spot). With cupric sulfate spray, the plate showed $R_f$ values of 0.37 for DPPE-PEG-amine and 0.49 for product (folic acid did not spot). A second plate sprayed with ninhydrin showed an $R_f$ value of 0.42 for DPPE-PEG-amine (no other spots were visible).

Analysis of the product by $^1$H NMR showed peaks for 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)].

Mass spectroscopy was performed with a quadrupole mass spectrometer. A peak at 786 mass-charge ($m/z$) ratio at a charge of 4 verified the molecular weight of the product was 3144 mass units. Mass spectra for the starting materials found a

Table 2
Summary of folate insertions into pre-formed liposomes

| Number of folate molecules intended for insertion per liposome (% of lipid formulation) | Average number of folate molecules inserted per liposome (% of lipid formulation) | Folate ligand insertion efficiency (%) |
|---|---|---|
| 30 (0.02%) | 30 (0.02%) | 100 |
| 75 (0.05%) | 75 (0.05%) | 100 |
| 150 (0.1%) | 145 (0.097%) | 90 |
| 300 (0.2%) | 280 (0.187%) | 93 |
| 500 (0.33%) | 500 (0.33%) | 100 |
| 750 (0.5%) | 695 (0.46%) | 92.7 |
| 1000 (0.67%) | 770 (0.51%) | 77 |
| 1250 (0.83%) | 1080 (0.72%) | 86.4 |
| 1500 (1.0%) | 1130 (0.75%) | 75 |

mass of 441 for folic acid and 2716 for DPPE-$PEG_{2000}$-amine. The chemical structure of DPPE-$PEG_{2000}$-folate is shown in Fig. 1.

DPPE-$PEG_{2000}$-folate was micellized by hydrating (with distilled-deionized water) the conjugate molecules previously dissolved in DMSO solvent. The resulting micelles were dialyzed three times against distilled—deionized water to remove the DMSO solvent and were then analyzed for folate content. Nearly 100% of the initial folate added to form the micelles remained after dialysis. DLS measurement of the DPPE-$PEG_{2000}$-folate micelles indicated that the average diameter of the micelles was 170 nm.

### 3.4. DPPE-$PEG_{2000}$-folate incorporation into pre-formed liposomes

In order to construct liposomal formulations bearing defined numbers of targeting ligands, the DPPE-$PEG_{2000}$-folate vesicles were incorporated into the lipid bilayer of pre-formed liposomes by the 'post-insertion' technique (see Fig. 3C). Following dialysis to remove DPPE-$PEG_{2000}$-folate that had not incorporated into the bilayer, the number of folate molecules per liposome was determined by measuring the UV absorbance at 285 nm. Table 2 shows the number of folate molecules intended for incorpora-

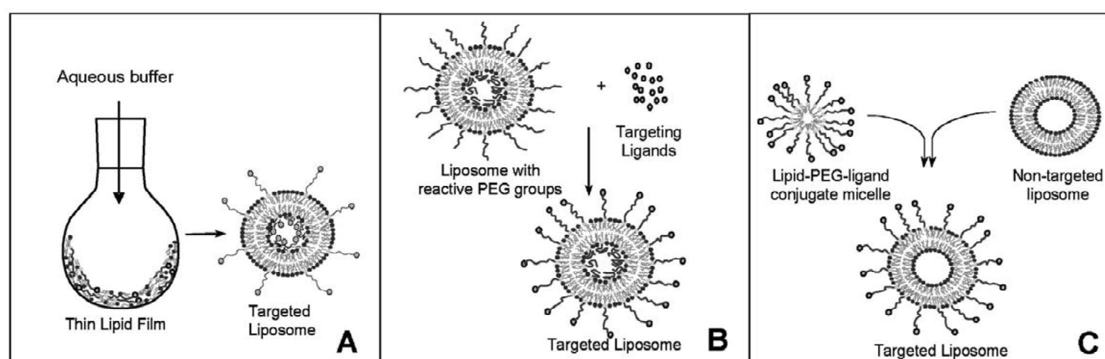



Fig. 3. Schemes for constructing targeted liposomes. Shown are three strategies for constructing targeted liposomes. (A) Traditional method of forming folate-targeted liposomes. A conjugate such as DPPE-$PEG_{2000}$-folate (see Fig. 1) is synthesized and included in the thin film lipid formulation for hydration into liposomes. (B) Method of forming targeted liposomes in which targeting ligands are coupled to the termini of functionalized PEG chains on liposomes. (C) Post-insertion technique by which a conjugate is micellized and incubated with pre-formed liposomes to yield targeted-liposomes. All targeting ligands are on the external leaflet of the liposomes and are available for targeting.

tion (first column) and the actual average number of molecules inserted as determined by UV absorbance (second column). As shown in Table 2, the number of ligands intended for insertion was between 30 and 1500 folate ligands per liposome. At higher ligand numbers (>500 folate ligands per liposome), the insertion efficiencies became less than unity, with the final range of targeting ligands falling between 30 and 1130. These values correspond to 0.02–0.75% of the total lipid formulation.

### *3.5. DOX loading and stability*

After DOX loading and dialysis to remove unencapsulated DOX, liposomal formulations were found to be stable for at least 24 h with no significant DOX leakage (data not shown). Stability of the liposomes ensures DOX did not leak from the liposomes and passively enter the cells.

### *3.6. DOX uptake in cells expressing different folate receptor profiles*

DOX in liposomal form is used clinically and has fluorescent properties, which allow its presence to be detected. DOX-loaded folate-targeted liposomes bearing defined numbers of targeting ligands were incubated with cells for 2 h. The amount of cell-associated DOX was then determined.

As shown in Fig. 4A,B, DOX uptake in both the KB and C6 cells increased up to 695 or 500 folate ligands per liposome, respectively. Beyond this saturation point, the uptake in these cell lines decreased. However, for the E9 chick cortical cells, the uptake increased over the full range of ligand numbers without the apparent saturation observed in KB and C6 cells. In order to determine if the DOX uptake was mediated by the folate receptor and not via non-specific binding events between folate liposomes and the cell surface, a competitive binding assay was performed. For these experiments, 1 mM free folic acid was added to the cell culture wells. This amount of folic acid is approximately a 1000-fold excess of the folate bound to the 300 folate liposomes and a 200-fold excess in the folate bound to the 1130 folate liposomes. Fig. 5 shows the results of the competitive binding assays for KB, C6, and E9 chick cortical cells with 300 folate targeting ligands per liposome. Because DOX uptake by folate liposomes bearing 300 targeting ligands was significantly reduced in KB and C6 cells when co-incubated with 1 mM free folic acid, a competitive binding experiment was also performed in C6 and KB cells when incubated with liposomes bearing 1130 folate targeting ligands, as shown in Fig. 6. Figs. 5 and 6 show that 1 mM free folic acid significantly reduced the DOX uptake in KB and C6 cells targeted with liposomes bearing either 300 or 1130 folate targeting ligands ($P<0.05$) but had no significant effect in reducing DOX uptake in cortical cells targeted with folate liposomes bearing 300 folate targeting ligands ($P=0.77$), as shown in Fig. 5.

### *3.7. Cellular DOX uptake under co-culture conditions*

In order to determine if folate-targeted liposomes would show specificity for folate-receptor expressing cells, co-culture experiments were performed in which two cell types were incubated in the same 35-mm diameter culture well separated by a physical barrier (see Fig. 2 for schematic of co-culture system). As shown in Fig. 7, when C6 cells were co-cultured with cortical cells, C6 cells took up more DOX with both conventional and folate targeted liposomes. The increased levels of uptake are comparable to the levels of increased uptake observed under single culture conditions. Similar results were obtained in the KB/cortical cell co-culture system where KB cells showed more uptake of DOX from both conventional and folate-targeted liposomes. The levels of DOX uptake of cortical cells from conventional and folate liposomes did not change significantly when cultured with either C6 or KB cells. Co-culture of C6 cells and KB cells showed that KB cells took up more DOX than C6 cells for both conventional and folate-targeted liposomes. The differences in uptake in the two cell types were comparable to those observed in the single culture systems. Again, C6 cells did not show significant difference in uptake when cultured with KB cells than when cultured with E9 cortical cells, and KB cells did not show significant differences in DOX uptake when cultured with C6 cells than when cultured with cortical cells.

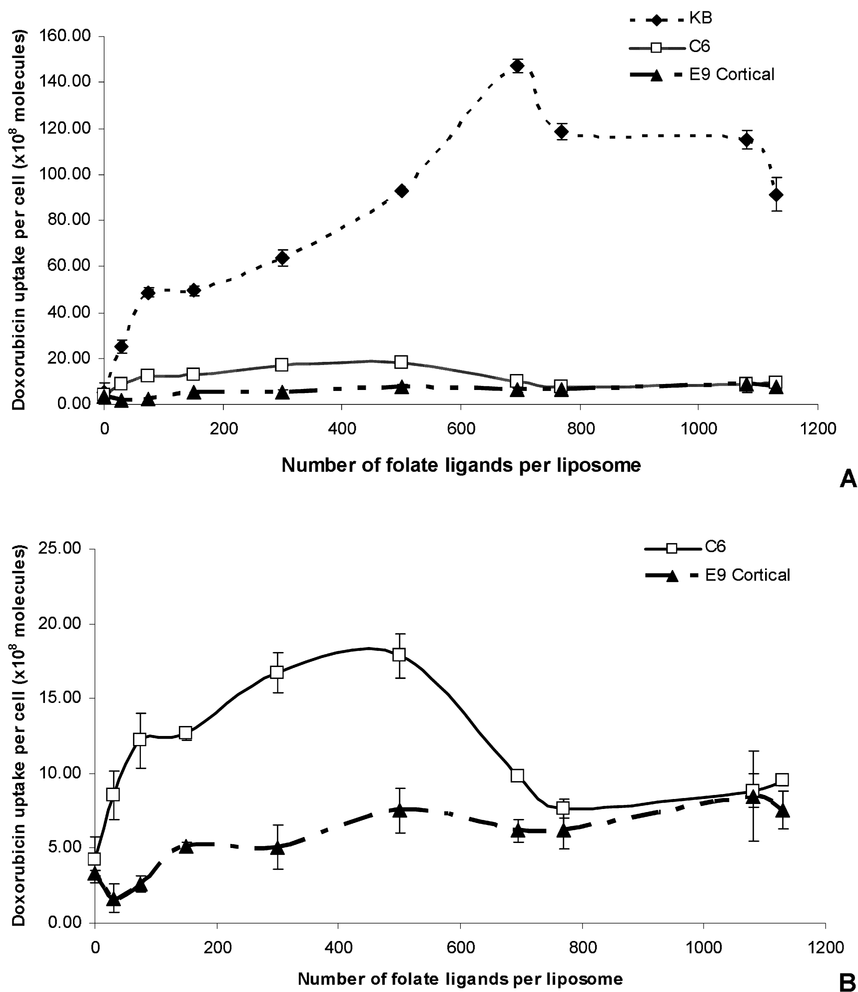



Fig. 4. DOX Uptake in three cell types as a function of number of folate targeting ligands per liposome. (A) Results for the three representative cell types tested (KB cells, C6 glioma cells, and E9 chick cortical cells) following 2-h incubation with 100 μg of DOX in liposomes bearing indicated number of targeting ligands per liposome. $n>3$ for all data points where $n$ is the result of one well of cells. $n>6$ for KB and C6 cells with 0–300 folate ligands. Error bars denote standard deviation. (B). Expanded view of cellular DOX uptake in C6 glioma and E9 cortical cells. Maximum differentiation in drug uptake between the two cell types is obtained at 300 folate ligands per liposome.

### *3.8. Cellular effect following incubation with conventional or folate liposomes*

Specific amounts of DOX must reach the nucleus of the cell to elicit the effect of the drug. In order to determine if sufficient amounts of DOX were reaching cellular nuclei to lead to cell death or stop cellular proliferation, an experiment was performed in which cells were either left untreated or subjected to a 2 h incubation with 10 μM DOX in conventional or folate-targeted liposomes bearing 300 folate molecules each. A total of 500 000 cortical cells was cultured as described above for 96 h on collagen coated dishes while 50 000 KB and C6 cells were cultured for 40 h prior to DOX treatment. Although cell numbers were different, the densities of the cells appeared similar due to differences in cell sizes and morphologies (see Fig. 8A). Immediately following DOX treatment, all wells of a given cell type appeared similar in cell density and morphology,

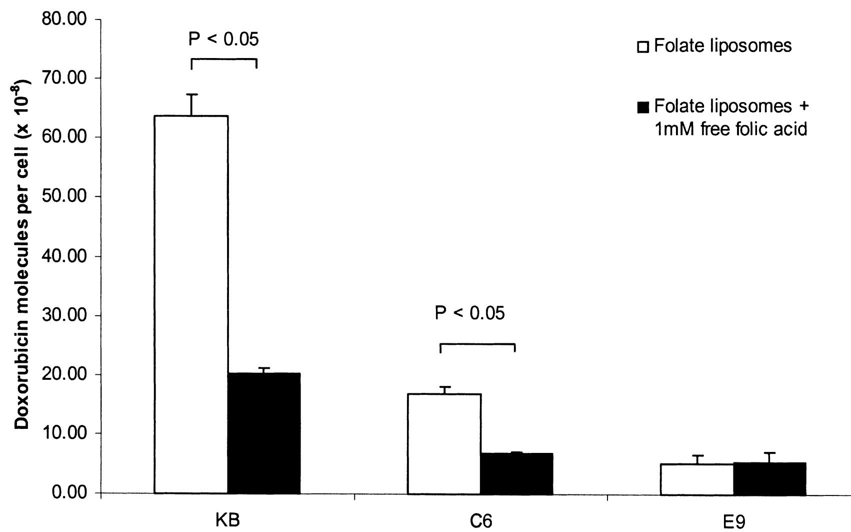



Fig. 5. Competitive binding assay in three cell types with 300 folate targeting ligands per liposome and 1 mM free folic acid. KB cells showed a 78% reduction in the amount of cell-associated DOX when incubated with 1 mM free folic acid. C6 cells showed a reduction of 58% in cell-associated DOX uptake. E9 cortical cells showed no reduction in cell-associated DOX. $n>3$ for all experiments and $n>6$ for KB and C6 cells where $n$ denotes results from one well of cells. Error bars denote standard deviation.

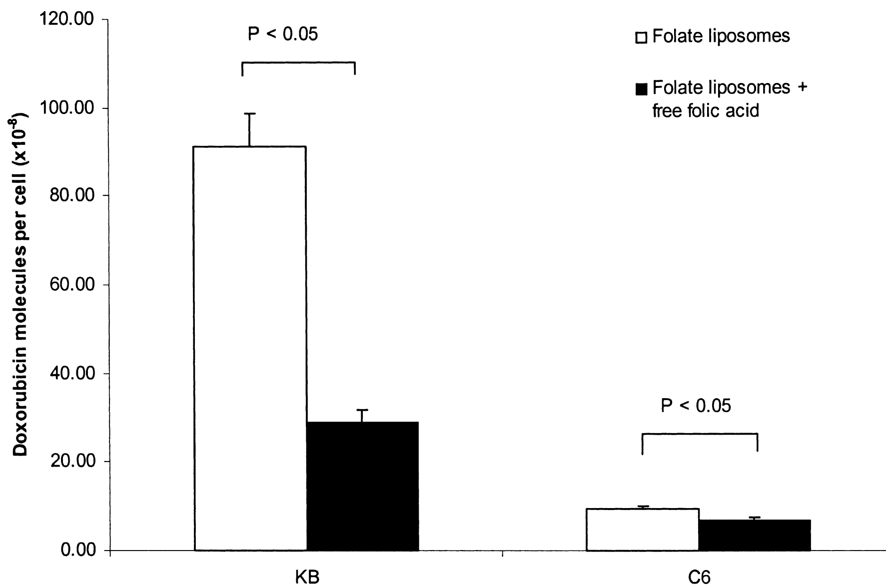



Fig. 6. Competitive binding assay in KB and C6 cell lines with 1130 folate targeting ligands per liposome and 1 mM free folic acid. KB cells showed a reduction in cell-associated DOX of 68% when incubated with 1 mM free folic acid while C6 cells showed a reduction of 28% relative to cells not competitively inhibited by free folic acid. $n=3$ for each bar where each $n$ is the result of a single well from one experiment. Error bars denote standard deviation.

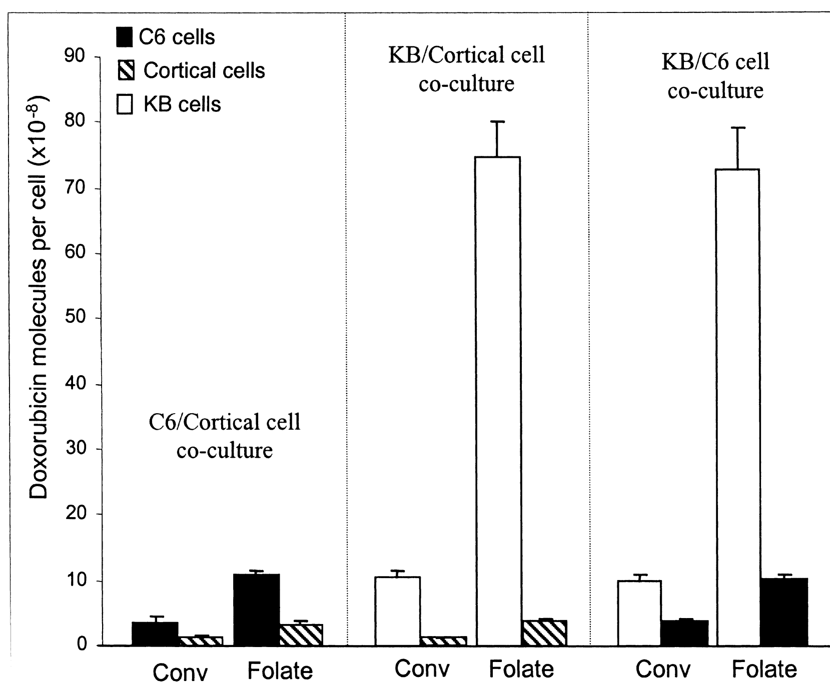



Fig. 7. Co-culture uptake of liposomal DOX in conventional or folate-targeted liposomes. Two cell types were placed in a single culture well and shared medium containing DOX in conventional or folate liposomes bearing 300 folate targeting ligands. Each combination of cells was tested. Results show that cellular DOX uptake was not affected by the presence of another cell type.

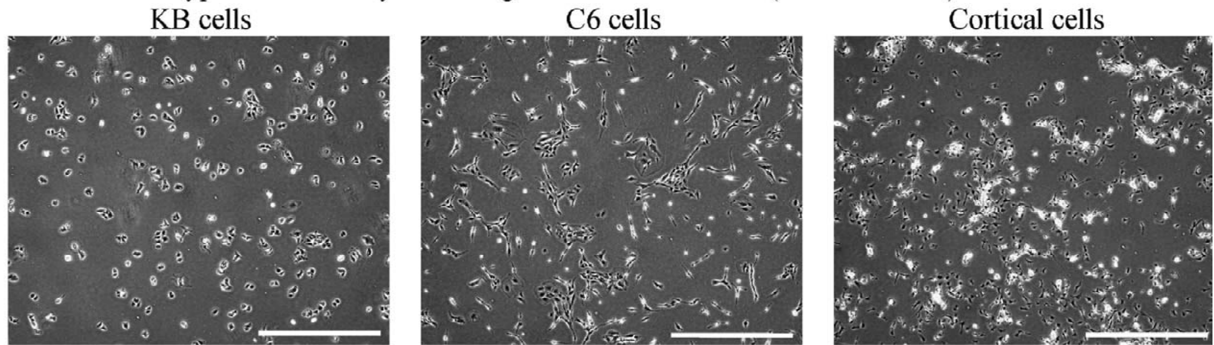
indicating that the treatment itself did not affect the densities in a given well and indicating that the treatment did not have an immediate effect. Cells were then observed over the next 6 days. Note that DOX was removed from the cells following an initial 2 h incubation and that cells were incubated for 6 days in DOX-free cell culture medium.

Fig. 8A shows representative phase contrast images of each cell type immediately following treatment ($t=0$ h). Fig. 8B shows phase contrast images of the cell types at 144 h following treatment. KB cells receiving no treatment showed significant proliferation and were nearly confluent. KB cells treated with conventional liposomes had also proliferated considerably, but to a lesser extent than untreated cells. However, by 144 h, KB cells treated with folate liposomes had lifted from the plate and had not proliferated. C6 cells left untreated were fully confluent and tightly packed (i.e., overgrown). C6 cells treated with conventional liposomes had also proliferated and were nearly confluent. However, C6 cells treated with folate liposomes were considerably less confluent than untreated or conventional liposome-treated cells and appeared not to proliferate following observation at 48 h following the single DOX treatment. Cortical cells treated with liposomal DOX were less confluent than untreated cells. However, no difference was observed between cortical cells treated with conventional or folate-targeted liposomal DOX

## 4. Discussion

Tumor cells are known to exhibit considerable variation in the number and types of receptors that they over-express relative to healthy tissues and relative to other types of tumors [9,22,23]. The over-expression of a given receptor is often used to target drugs and/or drug delivery vehicles such as liposomes to tumor cells both in vitro and in vivo [2,10,21,24]. For cells such as KB cells and HeLa

A. Three cell types immediately following two hour incubation (time = 0 hours).




B. Three cell types at 144 hours following two hour incubation (time = 144 hours).

KB cells no treatment | C6 cells no treatment | Cortical cells no treatment

KB cells conv liposomes | C6 cells conv liposomes | Cortical cells conv liposomes

KB cells 300FA liposomes | C6 cells 300FA liposomes | Cortical cells 300FA liposomes

Fig. 8. Effect of liposomal doxorubicin on cells. Cell death and proliferation were observed in KB, C6, and E9 cortical cells following no treatment or a two-incubation with 10 μM DOX encapsulated in conventional (conv liposomes) or folate-targeted liposomes bearing 300 folate targeting ligands per lipsome (300FA liposomes). (A) Cells immediately following two-incubation. (B) Cells 144 h after treatment.

cells that vastly over-express the folate receptor, the use of the folate targeting ligand mediates uptake of a variety of drugs and anti-tumor agents via the folate receptor [1–3,7]. However, the possibility of enhancing uptake of drugs and anti-tumor agents via the folate receptor in cells with lower levels of folate receptor expression may provide a treatment modality in systems where targeted cells express the receptor in the presence of surrounding tissues bearing none of the targeted receptor. One such situation is that of glioma of the brain, in which tumor cells are surrounded by neural tissue. Gliomas have been shown to express the folate receptor, but at lower levels than in other types of tumors [9]. However, healthy neural tissue does not express the receptor. Prognosis is poor in patients with this disease, and new methods of targeting and treatment which preferentially kill tumors or retard growth would be a useful improvement.

Three cell types which show different levels of folate receptor expression were selected for this study, as determined by [$^{3}$H]folic acid radiolabeling assays. The KB cell line has been widely used for studying the role of the folate receptor in targeted drug uptake due to its known over-expression of the folate receptor. The C6 glioma cell line is a rat glioma cell line that has been used for a variety of in vitro and in vivo experiments to study brain tumors [21,25,26]. It was found that C6 cells express the folate receptor, although at levels less than in KB cells (~30-fold decrease in receptor expression). E9 chick cortical cells were selected as representative cells for normal or 'healthy' tissue. When dissociated and cultured on a collagen matrix, the cells were found to have no detectable folate receptors on their surface (see Table 1). This result suggests that these cells are comparable to normal brain tissue that might be encountered in vivo.

In order to determine the optimal point of drug uptake enhancement between C6 glioma and cortical cells, liposomes bearing a range of folate ligands were formulated from a single batch of liposomes. Until recently, fine control over the number of ligands present at the liposome surface was not possible from a single preparation of liposomes. Rather, multiple preparations of various targeting ligands were required. This was previously done by two methods. As shown in Fig. 3A, the DPPE-PEG$_{2000}$-folate (or other small molecule conjugates) can be included in a thin lipid film prior to liposome formation [1]. This method would require multiple preparations and multiple extrusions to achieve the range of ligand formulations achieved in this study from a single preparation. Additionally, approximately half of the targeting ligands incorporate to the inner part of the liposome, making them unavailable for targeting. Fig. 3B shows the method of coupling the targeting molecule (which could be folate, small molecules, or macromolecules such as proteins and monoclonal antibodies) [10,21,24]. This method can utilize a single preparation of liposomes, but requires multiple coupling chemistries to be utilized (i.e., preparation of multiple formulations and reactions). Unfortunately, control of the ratios of functional PEG groups, targeting ligands and the cross-linkers as well as batch-to-batch variation makes tight control over number of targeting ligands per liposome difficult.

However, by the method shown in Fig. 3C [11–13], this study demonstrates that control over folate ligand numbers from a single preparation of liposomes is possible. This was done by the 'post-insertion' technique in which different amounts of micellized DPPE-PEG$_{2000}$-folate conjugate were incubated with the preformed conventional liposomes to give liposomal formulations bearing between 0 (conventional liposomes with no folate ligands) and 1130 folate ligands per liposome.

As shown in Table 2, the insertion of the DPPE-PEG$_{2000}$-folate conjugate was efficient at lower ligand numbers. Only at the higher number of targeting ligands (750–1500 targeting ligands) was the intended number of targeting ligands much less than the actual number inserted, suggesting that the 'post-insertion' method is viable for folate targeting.

The folate conjugate itself was synthesized by methods similar to those previously published [3]. Synthesis of the conjugate was confirmed by TLC, $^{1}$H NMR, and mass spectroscopy. TLC of the product showed an $R_f$ value of 0.49 under both UV (shows presence of folate) and with cupric sulfate (shows presence of phospholipids). The $R_f$ for the product was different than the $R_f$ of either starting material. Spots for free folic acid or DPPE-PEG$_{2000}$-amine starting materials were not observed in the product lane, suggesting minimal impurity. A sepa-

rate plate with product was negative for ninhydrin spray (free amine), indicating the formation of a bond between the amine of DPPE-PEG$_{2000}$-amine and the carboxyl of folic acid. $^1$H NMR and mass spectroscopy both confirmed the synthesis of the conjugate. $^1$H NMR showed peaks characteristic of the presence of both DPPE-PEG$_{2000}$ and folic acid in stoichiometric amounts for the conjugate. Mass spectroscopy confirmed the presence of a product of molecular weight at 3140 Da, near the theoretical molecular weight of DPPE-PEG$_{2000}$-folate.

The folate conjugate was inserted into pre-formed liposomes, which were then loaded with the anticancer drug DOX for targeting the three cell types. As shown in Fig. 4, each cell type took up very little DOX when no folate targeting ligands were attached. As expected, KB cells took up large amounts of DOX even at low ligand numbers (30 and 75 folate targeting ligands). Maximal uptake in KB cells occurred with 695 folate targeting ligands, beyond which point a saturation effect occurred. C6 cells were also found to take up DOX in a manner dependent upon the number of folate targeting ligands, with maximum uptake occurring with 500 targeting ligands per liposome. This result is particularly significant in that although C6 cells exhibit nearly 30-fold fewer folate receptors on their cell surface, the amount of DOX uptake was only reduced by 5–10-fold, depending on the number of targeting ligands utilized. Unlike KB and C6 cells which showed saturable uptake of DOX, cortical cells showed variable but increasing amounts of DOX uptake over the range of ligands tested.

The DOX uptake as a function of increasing numbers of targeting ligands for the cortical cells was progressively higher. Because these cells have no detectable folate receptor on the cell surface, the steady increase of DOX uptake over the range of ligands tested was likely due to increasing amounts of non-specific binding with increasing numbers of targeting ligands. However, while the saturable behavior observed in KB and C6 cells was expected, the sharp decline in the amount of cellular DOX uptake at high ligand numbers was unexpected. This sharp decline may be a combination of limitations in the rate of liposomal uptake and the high number of internalized folate molecules leading, possibly, to a down-regulation or 'shut-off' of the folate receptor recycling system. Others have suggested that the amount of intracellular folate, in the range of $2\times10^7$ [27] to $9\times10^7$ [2] folate molecules per cell, may be responsible for the saturation and shut-off of the folate receptor uptake pathway. Therefore, the folate ligands conjugated to the liposomes may contribute to the intracellular folate concentration and lead to shut-off of the pathway. Liposomes bearing more folate targeting ligands would lead to larger intracellular folate content than those with less targeting ligands. Combined with the rate of uptake of the liposomes, this could lead to fewer liposomes being taken up when more targeting ligands are utilized, thus decreasing the cell-associated DOX.

Differences in the numbers of folate receptors and mechanisms of the folate receptor pathways in KB and C6 cells could account for the difference in the location of the peak in the two cell types. The peak for KB cells occurred at 695 folate targeting ligands per liposome while the peak for C6 cells occurred at 500 targeting ligands. The discrepancy in the peak location and in the shape of the uptake curves also suggests differences in the cells rather than a discrepancy in the liposomal formulation.

An alternative explanation to the decrease in DOX uptake at higher ligand numbers in KB and C6 cells is a possible limitation on the 'post-insertion' method of the folate conjugates. The diameter of liposomes is 130 nm while the average diameter for the DPPE-PEG$_{2000}$-folate micelles was 170 nm, making size-based separation of the two components difficult. Because liposomes bear the folate conjugate, separation by affinity techniques is also questionable. Therefore, significant incorporation of the folate conjugate into the liposomes was relied upon. This would drop the DPPE-PEG$_{2000}$-folate conjugates below their critical micelle concentration (CMC), leading to non-micellized conjugates (MW~3140). Dialysis against the 300 000 MWCO membrane would then easily remove the DPPE-PEG$_{2000}$-folate conjugate (MW~3140 Da). However, if insertions became inefficient at higher number of targeting ligands and the micelles did not fall below their CMC (~10 μM for DPPE-mPEG$_{2000}$), stable micelles would remain that would not be dialyzed from the liposomal formulation. This would set up a competitive binding scenario in which DPPE-PEG$_{2000}$-folate micelles would compete with the

folate receptors, preventing folate-liposome binding and uptake.

This explanation may not fully explain the decrease in the amount of cellular DOX uptake, particularly in C6 cells. The amount of DOX uptake in the range of 695–1130 folate ligands decreases nearly to the levels observed in the competitive binding experiment with liposomes bearing 300 folate targeting ligands in the presence of 1 mM free folic acid (see Fig. 5), a 1000-fold excess of free folic acid. The amount of non-liposomal DPPE-$PEG_{2000}$-folate in micellar form would be orders of magnitude less than this amount of free folic acid. However, the multi-valency of the micelles (i.e., many folic acid ligands in the micelle) would allow a single micelle to occupy multiple receptors, thus increasing the binding affinity and helping to account for the decrease in cellular DOX uptake at high ligand numbers.

The difference in the uptake behavior in KB and C6 cells also indicates a role for the folate receptor recycling pathway as a possible explanation for the sharp decrease in DOX uptake at higher ligand numbers for KB and C6 cells. Additionally, the use of the 'post-insertion' technique is not compromised significantly if decreased amounts of micelle insertion and a competitive binding scenario are responsible for the decrease in DOX uptake at high ligand numbers because the numbers of targeting ligands where the drop occurs is 2–3-fold greater than the number of folate targeting ligands previously utilized by others for folate targeting of liposomes [1,2] and than the optimal number of targeting ligands determined in these experiments to maximally differentiate uptake between C6 and E9 cortical cells. In other experiments when cells were cultured under similar conditions, cellular uptake by folate liposomes bearing 75 targeting ligands formed by the 'post-insertion' method showed higher uptake than when the conjugate was included in the lipid formulation (results not shown). This again indicates the utility of the 'post-insertion' technique.

The increasing uptake over the range of ligands tested in cortical cells underscores the importance of choosing the optimal number of targeting ligands in differentiating uptake between tumor cells expressing low levels of receptor (C6 cells) and healthy cells (E9 cortical). At high numbers of targeting ligands (675–1130 per liposome) DOX uptake in C6 cells and E9 cortical cells were not significantly different. However, significant differentiation in drug uptake was easily obtained when 75–300 targeting ligands per liposome were utilized (see Fig. 4B).

The dependence of uptake on the folate receptor in KB and C6 cells was established by performing a competitive binding assay in the presence of 1 mM free folic acid. This amount of free folic acid is ~1000-fold higher than the amount of folate introduced to the system by liposomes bearing 300 folate ligands per liposome and ~200-fold higher than the amount of folate introduced by liposomes bearing 1130 targeting ligands. The high concentration of free folic acid necessary to competitively inhibit folate receptor-dependent binding and uptake has previously been postulated to be due to the multiple interactions between folate receptors and folate targeting ligands on the liposome, which leads to an exponential effect on the binding affinity between ligand and receptor (i.e., so-called avidity effect).

As shown in Figs. 5 and 6, 1 mM free folic acid reduced folate-mediated liposomal DOX uptake in KB cells by 78% when 300 targeting ligands were used and 68% when 1130 targeting ligands were utilized. Although C6 cells exhibited significantly less uptake than KB cells, Figs. 5 and 6 show that this uptake was also dependent on the folate receptor. 1 mM free folic acid reduced uptake in C6 cells by 60% when 300 folate targeting ligands were used and by 28% when 1130 targeting ligands were used. This decrease is statistically different in both cases ($P<0.05$). Unlike KB and C6 cells, DOX uptake in cortical cells was not found to be dependent on the folate receptor when liposomes bearing 300 targeting ligands were used (see Fig. 5), as the inclusion of 1 mM free folic acid did not affect the amount of cellular DOX uptake. Therefore, the DOX uptake in cortical cells appears to be due to non-specific binding at the cell surface.

The results of these experiments show that in the case where the folate receptor is vastly over-expressed (KB cells), differentiating drug uptake between cells with little (C6) or no (cortical cells) is easily accomplished, even at low ligand numbers. However, this study also shows that in the case

where the folate receptor is expressed even in low numbers on the cell surface (C6), significant differentiation can be achieved between cells that have no cell surface folate receptors (cortical cells). In order to achieve this differentiation between C6 and cortical cells, however, the number of targeting ligands becomes important. If too few ligands are used, the uptake in the C6 cells is not sufficient to elicit the desired response, which in vivo would be cell death. If too many targeting ligands are used, the non-specific binding to the healthy cells (cortical cells) becomes too large to achieve differentiation with the tumor cells. Thus, there exists an optimal number of targeting ligands at which significant drug uptake occurs in the tumor cells while the uptake in the healthy cells is still low (little non-specific binding). In these experiments, the optimal ligand number for differentiating between C6 and cortical cells occurred with 300 folate targeting ligands per liposome.

In order for this differentiation to be clinically relevant, the differentiation must also be observed when the cells are present together. That is, it is necessary for the folate liposomes to be preferentially taken up by the tumor cells in the presence of healthy cells. In order to confirm that this is the case, co-culture experiments were performed in which one cell type (e.g., KB cells) was co-cultured with one of the other cell types (e.g., C6 glioma or cortical cells). This was accomplished by placing a physical barrier between the two cell types, thus allowing the different cell types to be examined individually for DOX content after sharing the same culture medium. All cells were washed three times with PBS, lysed, and analyzed separately from other cells for DOX content. As shown in Fig. 7, the results indicate that the ratios of DOX uptake observed with single culture were maintained in the co-culture system. Additionally, the amount of DOX uptake observed in each cell type did not vary depending on the other cell type in the co-culture. This is likely because the DOX concentration for these experiments is such that the cells can take up liposomal drug without significantly depleting the amount of DOX in the extracellular volume of the well. That is, the DOX concentration is above the saturation levels of the cells, allowing both cell types in co-culture to take up the maximal amount of drug. Thus, at the DOX concentrations studies in these experiments, the folate liposomes maintain selectivity for cells expressing folate receptor in the presence of cells without the folate receptor (cortical cells).

While differentiation in DOX uptake is necessary between tumor and healthy cells, it is also imperative that the amount of drug taken up by the tumor cells be sufficient to elicit the appropriate response (i.e., cell death or reduced rate of proliferation) while the amount of drug taken up by healthy cells must remain low enough to avoid toxicity to these cells. Therefore, an experiment was performed in which cells were either left untreated or were subjected to a 2-h incubation with liposomal DOX encapsulated in conventional or folate-targeted liposomes, as shown in Fig. 8. Fig. 8A shows representative images of cells immediately following treatment. No differences were observed between wells following the treatment, indicating that the treatment procedure itself did not affect the cells. Fig. 8B shows that by 6 days following the initial 2-h incubation with DOX, KB cells treated with folate-targeted liposomal DOX were nearly completely dead. It was found that C6 cell proliferation was obviously delayed by treatment with folate-targeted liposomes compared to C6 cells left untreated or those subjected to conventional liposomes. Lastly, it was observed that although treatment with liposomal DOX appeared to decrease cell density in cortical cells compared to untreated cells, the effect was independent of folate-targeting. These results further indicate that folate targeting of glioma cells bearing low numbers of folate receptors is a viable option when surrounded by healthy cells bearing no folate receptor. The qualitative nature of these results was largely due to an inability to reliably count the cells due to cell density and morphology. Nonetheless, the results shown in Fig. 8B are dramatic. Further, these results are following a single 2-h treatment with liposomal-DOX. Most in vivo treatment regimens utilize multiple dosages, presumably leading to better tumor cell kill. These results indicate that even one treatment with folate targeted liposomes can kill KB cells efficiently and greatly slow or stop the rate of C6 cell proliferation despite low numbers of the targeted folate receptor.

Because liposomal formulations have been shown

to extravasate to brain tumors via the enhanced permeability and retention (EPR) effect [28], the enhanced uptake of DOX at an optimal ligand number may provide critical sparing of healthy tissue and increased uptake of drug at the tumor in vivo.

## 5. Conclusions

A DPPE-PEG$_{2000}$-folate conjugate was successfully synthesized. This study shows, for the first time, that this conjugate can be inserted into pre-formed liposomes bearing an ammonium sulfate gradient for DOX loading. This 'post-insertion' method provides the ability to formulate a range of ligand numbers from a single batch of liposomes. This study also shows that when cells bear some number of folate receptors, a saturable effect occurs in DOX uptake as a function of the number of folate targeting ligands. However, when cells do not express surface folate receptors, the drug uptake increases due to non-specific binding events. Thus, tumor cells with low levels of folate receptor expression can still achieve significant uptake of DOX mediated by folate liposomes compared to healthy cells bearing no folate receptor. The differential uptake between the two cell types can be optimized by choosing the appropriate number of targeting ligands. In C6 glioma cells, it was found that the amount of DOX uptake was sufficient to retard the growth of the cells, while incubation with the same formulation of DOX-loaded folate liposomes had minimal effect on healthy primary cortical cells. Because Stealth® liposomes have been shown capable of extravasating across the blood–brain barrier to brain tumors in vivo [28], this method may prove to be a valuable method of increasing targeting to brain tumors. Our laboratory is currently pursuing folate liposome-mediated uptake of DOX in an in vivo C6 glioma model to test this possibility.

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