

Intracellular uptake and intracavitary targeting of folate-conjugated liposomes in a mouse lymphoma model with up-regulated folate receptors

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Abstract

The folate receptor is overexpressed in a broad spectrum of malignant tumors and represents an attractive target for selective delivery of anticancer agents to folate receptor-expressing tumors. This study examines folate-lipid conjugates as a means of enhancing the tumor selectivity of liposome-encapsulated drugs in a mouse lymphoma model. Folate-derivatized polyethylene glycol (PEG₃₃₅₀)-distearoyl-phosphatidylethanolamine was post-loaded at various concentrations into the following preparations: radiolabeled PEGylated liposomes, PEGylated liposomes labeled in the aqueous compartment with dextran fluorescein, and PEGylated liposomal doxorubicin (PLD, Doxil). We incubated folate-targeted radiolabeled or fluorescent liposomes with mouse J6456 lymphoma cells up-regulated for their folate receptors (J6456-FR) to determine the optimal ligand concentration required in the lipid bilayer for liposomal cell association, and to examine whether folate-targeted liposomes are internalized by J6456-FR cells in suspension. Liposomal association with cells was quantified based on radioactivity and fluorescence-activated cell sorting analysis, and internalization was assessed by confocal fluorescence microscopy. We found an optimal ligand molar concentration of ~0.5% using our ligand. A substantial lipid dose-dependent increase in cell-associated fluorescence was found in folate-targeted liposomes compared with nontargeted liposomes. Confocal depth scanning showed that a substantial amount of the folate-targeted liposomes

are internalized by J6456-FR cells. Binding and uptake of folate-targeted PLD by J6456-FR cells were also observed *in vivo* after i.p. injection of folate-targeted PLD in mice bearing ascitic J6456-FR tumors. The drug levels in ascitic tumor cells were increased by 17-fold, whereas those in plasma were decreased by 14-fold when folate-targeted PLD were compared with nontargeted PLD in the i.p. model. Folate-targeted liposomes represent an attractive approach for the intracellular delivery of drugs to folate receptor-expressing lymphoma cells and seem to be a promising tool for *in vivo* intracavitary drug targeting. [Mol Cancer Ther 2006;5(4):818–24]

Introduction

A variety of different targeting strategies are currently under investigation to enhance the specificity of antitumor drug carriers, including cytokines, antibodies, and growth factors. The vitamin folic acid has also shown potential as a targeting device because the folate receptor, a 38 kDa glycosyl-phosphatidylinositol-anchored glycoprotein, is highly overexpressed in a number of human tumors including ovarian (1), lung, brain, head and neck, renal cell, and breast cancers (2), whereas in normal tissue, its expression is significantly lower and limited mainly to kidney tubuli, lung epithelium in the apical (luminal) cell pole, the choroid plexus, and placenta for folate transport to the central nervous system and to the fetus (3).

The use of folate ligands as a targeting device provides a number of important advantages over other targeting ligands. They are inexpensive, nontoxic, nonimmunogenic, easy to conjugate to carriers, retain high binding affinity, and are stable in storage and in circulation (4). They can be conveniently post-loaded into previously prepared (preformed) liposomes (5). Folate-targeted drug carriers and imaging agents have shown selective binding and uptake in KB head and neck carcinoma and HeLa cells (6). Folate targeting has also improved gene delivery via cationic liposomes into cultured KB cells (7–9), as well as disseminated peritoneal tumors (10). Saul et al. (11) showed enhanced uptake of FTL in rat brain astrogloma tumors (C6) with minimally elevated levels of folate receptor expression compared with normal surrounding brain tissue. In another recent study, retinoic acid-induced folate receptor expression results in enhanced uptake of folate targeted liposomal doxorubicin into acute myeloid leukemia cells (12).

Receptor-mediated endocytosis of FTL has been shown in cultured human carcinoma cells (6, 13) based on fluorescent microscopy of calcein-loaded liposomes, and fluorescence spectrometric measurement of cell extracts. Our own work

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(14) has also shown the enhancement of doxorubicin uptake using doxorubicin-loaded FTL in M109 multidrug-resistant mouse carcinoma cells expressing folate receptor, indicating that folate receptor-mediated uptake can apparently bypass P-glycoprotein-mediated drug efflux. Folate receptor-mediated uptake apparently channels to a nonlysosomal compartment of acidic endosomes (15, 16), possibly reducing the risk of degradation of lysosomal-sensitive delivery materials such as proteins and nucleic acids.

Here, we compared the cellular association and internalization of liposomes with a range of different concentrations of our folate conjugate in the J6456 T-cell lymphoma tumor model up-regulated for the folate receptor (5). Unlike the previous epithelial tumor models that we have used to study folate liposome targeting, J6456 cells are lymphoid and grow in suspension, and provide a convenient *in vitro* and *in vivo* tumor model (17). Greatly enhanced fluorescence in the intracellular compartment was shown *in vitro*. We also examined the *in vivo* targeting of a folate-targeted pegylated liposomal doxorubicin formulation (PLD, Doxil) after i.p. injection in mice bearing the ascitic J6456 lymphoma and found compelling evidence of targeting in this intracavitary model.

Materials and Methods

Liposomal Preparations

Liposome formulations were prepared by standard methods of thin lipid film hydration and polycarbonate membrane extrusion through 0.05 μm pores as reported previously (18). Hydrogenated soybean phosphatidylcholine was obtained from Avanti (Birmingham, AL) or Lipoid (Ludwigshafen, Germany); cholesterol was purchased from Sigma (St. Louis, MO), and mPEG (2000)-DSPE was a gift from ALZA Corporation (Mountain View, CA). Folate-derivatized polyethylene glycol (PEG, 3350)-DSPE, provided by Dr. Samuel Zalipsky from ALZA, was synthesized in Dr. Zalipsky's laboratory as previously described (18). FTLs were composed, on a molar ratio basis, of 55% hydrogenated soybean phosphatidylcholine, 5% mPEG (2000)-DSPE, 40% cholesterol, and post-loaded with various concentrations of folate-PEG (3350)-DSPE. Liposomes were suspended in dextrose 5% buffered with 15 mmol/L Hepes (pH 7.0).

^3H -cholesterol radiolabeled liposomes were prepared by the addition of ^3H -cholesterol-hexadecyl ether (^3H -CHE; Amersham, Buckinghamshire, England) at a specific ratio of $\sim 0.5 \mu\text{Ci}/\mu\text{mol}$ phospholipid during liposome preparation. The use of ^3H -CHE is convenient for these studies because it is a stable, nonexchangeable, and nondegradable marker of liposomes (19); thus, allowing faithful determination of cell-associated liposomes.

Dextran-fluorescein (D-FITC; Sigma, molecular weight, $\sim 4,000$) labeled liposomes were prepared by resuspension of lyophilized lipids in 15 mmol/L HEPES buffer (pH 7.0) containing 30 mg/mL (3%) D-FITC at 60°C and bath-sonicated until all lipid dispersed and liposomes formed. Liposomes were extruded serially as described and then

dialyzed against buffer without D-FITC for up to 10 days at room temperature until the dialysis buffer was fluorescein-free as measured by fluorescent detection at $490_{\text{ex}}/520_{\text{em}}$ in a Kontron SFM-25 fluorimeter.

Ligand post-insertion was achieved by incubation of preformed liposomes with folate-derivatized PEG (3350)-DSPE at 45°C for 2 hours, at the desired concentrations. In the standard procedure, folate-PEG (3350)-DSPE was added at the ratio of 0.5% of phospholipid measured concentration (based on phosphorus content) to obtain FTLs. Liposomes were then centrifuged at 3,000 rpm for 10 minutes to remove nonincorporated ligand. The percentage of ligand incorporation was determined spectrophotometrically by measuring folate at 285 nm after liposome solubilization in isopropanol. Nontargeted liposomes contain mPEG (2000)-DSPE but no folate-PEG-DSPE. In some of the experiments, a formulation of PLD, known commercially as Doxil (provided by ALZA) was used with or without post-loading of folate-PEG-DSPE.

All formulations were analyzed for phosphorus content by the Bartlett method (20), folate content (OD, 285 nm), radioactivity (measured in a liquid scintillation Kontron counter) and vesicle size (measured by dynamic light scattering in a Coulter counter Model N4SD). Final phospholipid concentration was $\sim 40 \mu\text{mol}/\text{mL}$; folate content and ^3H -CHE cpm/mol phosphorus were close to the relative input preparation ratios; mean vesicle size was in the range of 70 to 90 nm with SD $< 30\%$ of the mean.

Tissue Culture

J6456-FR lymphoma cells were grown in folate-free RPMI (Beit Haemek, Israel) containing 10% calf serum and 10 $\mu\text{mol}/\text{L}$ mercaptoethanol. Based on studies with radio-labeled free folate, these cells have ~ 10 million receptors per cell.¹

In vitro Uptake of Folate-Targeted Liposomes

Binding of ^3H -CHE liposomes containing various concentrations of folate to J6456-FR lymphoma cells *in vitro*: ^3H -CHE labeled liposomes containing 0%, 0.05%, 0.1%, and 0.5% folate-PEG-DSPE were incubated for 3 hours at 37°C with 10^7 cells. Each folate preparation was assessed at various concentrations of phospholipid (6–100 nmol/mL PL). Cells were washed $3\times$ with buffer, pelleted, and dissolved in 20 mL scintillating fluid (Quicksafe A, Zinsser) and counted.

Fluorescence-activated cell sorting (FACS) analysis of D-FITC-liposomes: J6456-FR lymphoma cells (10^7) were incubated *in vitro* for 3 hours, at 37°C , with nontargeted or folate-targeted, D-FITC-labeled liposomes. After incubation cells were washed $2\times$ with PBS, rinsed with 50 mmol/L ammonium chloride, washed again with PBS, and incubated at room temperature for 30 minutes with 3% paraformaldehyde. Fluorescence was measured by FACScan (Becton Dickinson) at 488_{ex} and 520_{em} , and data were analyzed using CellQuest software.

¹ Dr. Chris Leamon, Endocyte, personal communication.

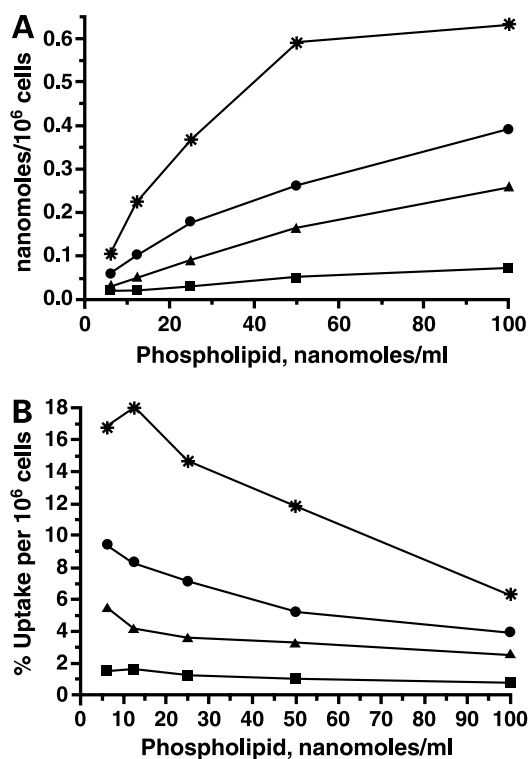


Figure 1. Uptake of folate-targeted radiolabeled liposomes into J6456-FR lymphoma cells. ³H-CHE radiolabeled liposomes containing 0% (■), 0.05% (▲), 0.1% (●), and 0.5% (*) folate-PEG-DSPE were incubated for 3 h at 37°C with 10⁷ J6456-FR lymphoma cells. Each liposome preparation was assessed at concentrations ranging between 6 and 100 nmol/mL of phospholipid and the cell-associated radioactivity was determined. **A**, absolute uptake, phospholipid nmol/10⁶ cells; **B**, relative uptake, percentage of liposome added/10⁶ cells.

Confocal fluorescence microscopy was done on J6456-FR lymphoma cells incubated with nontargeted and folate-targeted D-FITC-liposomes (0.5% folate ligand ratio) at various concentrations of phospholipid (0, 25, 50, 100, 200 nmol/mL PL) for 3 hours and then washed and incubated in regular media overnight (24 hr). For microscopy cells were fixed as for the FACS analysis then washed with PBS 1× and resuspended in 0.5 mL DABCO reagent and mounted on glass slides for observation using a Zeiss Model 410 confocal microscope (488_{ex}, 520_{em}).

In vivo Folate Targeting of PLD in the Peritoneal Cavity

To investigate the potential of folate liposome targeting for intracavitary cancer therapy, we used an ascitic tumor

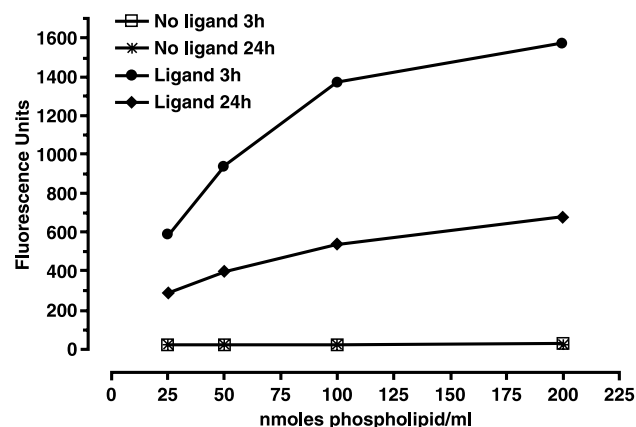


Figure 2. Cell association of folate-targeted, D-FITC-labeled liposomes to J6456-FR lymphoma cells. FACS analysis of cells (10⁷) incubated for 3 h with D-FITC liposomes containing 0.5% folate (●, ◆) compared with non-targeted controls (□, *). Cells were then washed and cultivated for 24 h in liposome-free fresh medium. Note that the decrease in fluorescence between the 3-h curve to the 24-h curve results from the dilution of the fluorescent label due to cell proliferation. The number of cells increased ~2-fold during the 24-h culture.

model and the i.p. route for liposome injection. We chose doxorubicin-containing liposomes to get a direct estimate of the *in vivo* targeting of a pharmacologic agent. PLD (Doxil, ALZA) was loaded with folate-PEG-DSPE at a molar ratio of 0.5%. J6456-FR cells (10⁶) were injected into the peritoneal cavity of BALB/c mice. Approximately 2 weeks after tumor inoculation, when ascites developed, 10 mg/kg of PLD or FT-PLD (five mice each) were injected i.p. in a volume of 0.2 mL. Sixteen hours later, mice were anesthetized, bled retroorbitally, and immediately sacrificed. The peritoneal cavity was rinsed with 3 mL PBS and the ascitic exudate removed, and centrifuged, separating the ascitic cells from the ascitic fluid. Plasma, ascitic peritoneal fluid and J6456-FR cellular doxorubicin levels were determined after extraction in acidic isopropanol and quantification of fluorescence as shown previously (21). In addition, samples of ascitic cells were fixed for confocal microscopy.

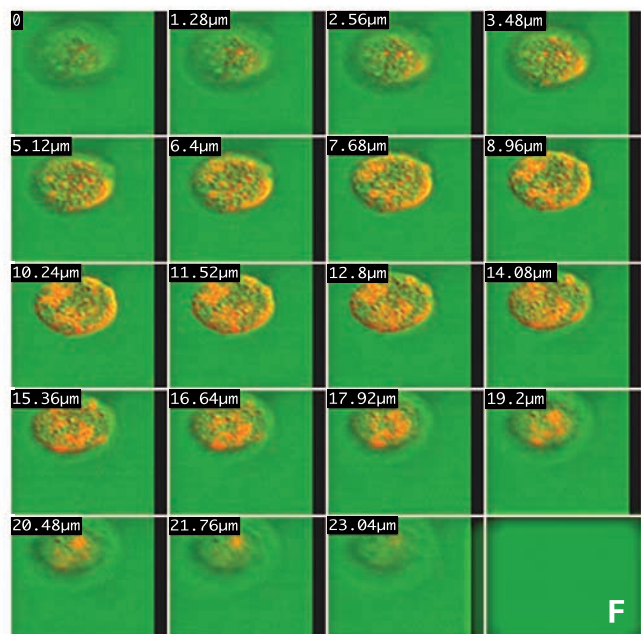
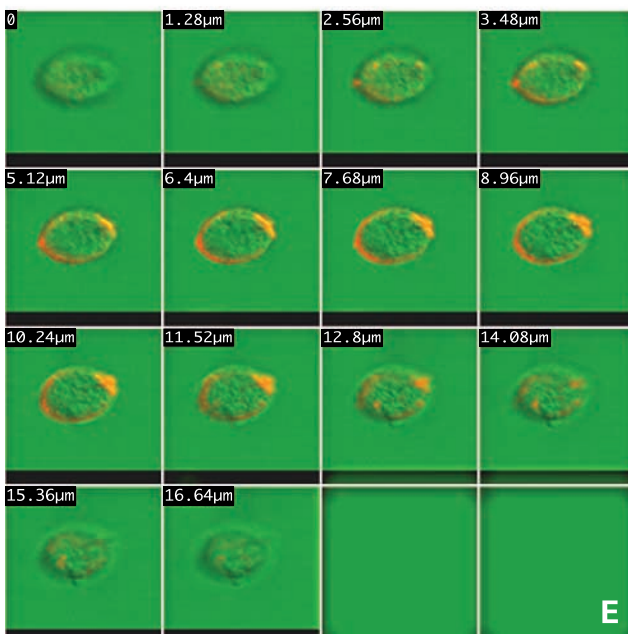
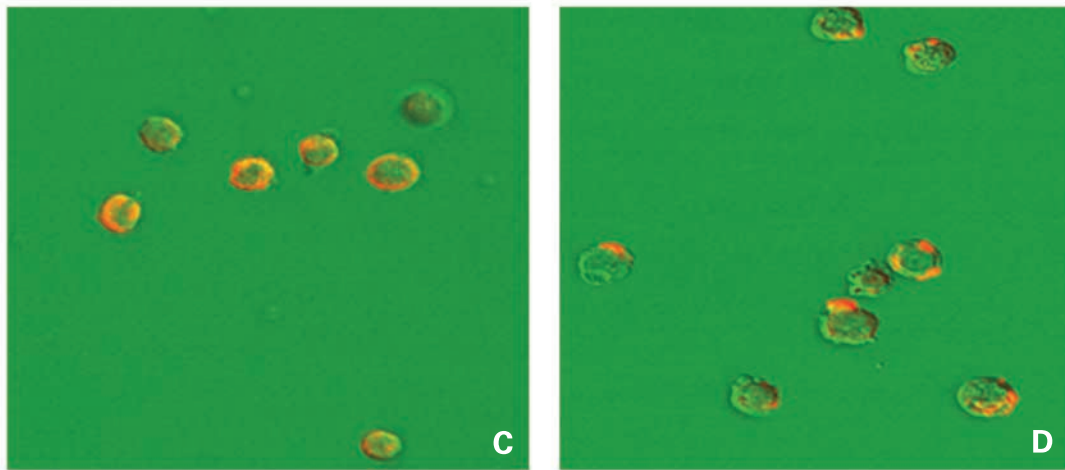
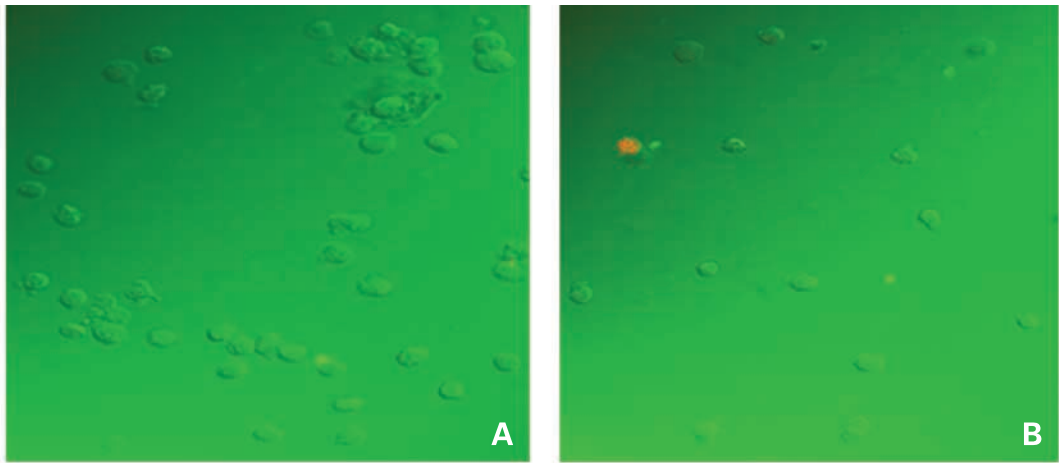
Results

Folate Uptake Based on ³H-CHE – Labeled Liposomes

³H-CHE-labeled liposomes post-loaded with various concentrations of folate were incubated for 3 hours with J6456-FR cells. The results in Fig. 1A and B show that under the conditions of our experiments, optimal cell association is achieved with 0.5% molar ratio of the folate

Figure 3. Confocal analysis of J6456-FR lymphoma cells exposed to folate-targeted D-FITC liposomes. Fluorescent confocal images of J6456-FR cells incubated with D-FITC liposomes containing 0.5% folate-PEG-DSPE, or with nontargeted D-FITC liposomes. Cells were incubated for 3 h with the liposome preparations, then washed and incubated in medium without liposomes for another 24 h. Cells were fixed as for the FACS analysis, washed with 1× PBS and then resuspended in 0.5 mL DABCO reagent and mounted on glass slides for observation using a Zeiss model 410 confocal microscope (488_{ex}, 520_{em}). **A**, control, 3 h of incubation; **B**, nontargeted D-FITC liposomes, 3 h of incubation; **C**, folate-targeted D-FITC liposomes, 3 h of incubation; **D**, folate-targeted D-FITC liposomes, 24 h of incubation; **E**, stepwise depth scan of folate-targeted D-FITC liposomes, 3 h of incubation; **F**, stepwise depth scan of folate-targeted D-FITC liposomes, 24 h of incubation.

Figure 3.



ligand. Cell association is dependent on the concentration of phospholipid and plateaus at ~ 50 nmol/mL. At lower concentrations of phospholipid, there is relatively more cell association suggesting a saturation phenomenon at lipid concentrations >25 nmol/mL (Fig. 1B). Calculation of the approximate number of liposomal particles associated per cell yields $\sim 40,000$ particles per cell (based on an average number of 10,000 phospholipid molecules per liposome).

Cell-Associated D-FITC – Labeled Folate-Targeted Liposomes in J6456-FR Lymphoma Cells

FACS analysis of cells incubated for 3 hours with D-FITC liposomes containing 0.5% folate show a 50- to 70-fold increase in cell-associated fluorescence compared with the nontargeted controls (Fig. 2). When these cells are washed after completion of the 3-hour incubation with liposomes and cultivated for 24 hours in liposome-free medium, 37% to 49% of the fluorescence remained cell-associated when measured in a fixed number of cells. This is expected because the cell number approximately doubled during this additional period of incubation (20–24 hours), thereby diluting the liposome marker in a larger cell population.

In vitro Internalization of Folate Targeted D-FITC – Labeled Liposomes Based on Confocal Microscopy

Confocal microscopy of cells incubated for 3 hours with D-FITC-labeled liposomes reveals a fluorescence pattern with either an even distribution around the periphery of the cell or a capped-like distribution (Fig. 3C). No fluorescence was detectable in cells incubated with nontargeted D-FITC liposomes (Fig. 3B). After removal of the medium containing fluorescent liposomes, and further incubation for 24 hours, the fluorescence distribution is located either in capped formations or in multifocal patches suggesting internaliza-

tion (Fig. 3D). Depth scan analysis with serial confocal microscopy slices of representative cells show membrane-attached, peripheral fluorescence at 3 hours of incubation (Fig. 3E), and intracellular fluorescence at 24 hours of incubation (Fig. 3F), confirming liposome internalization.

In vivo Folate-Targeting of Doxil (FT-PLD) in the Peritoneal Cavity

The potential of FTL to selectively associate with J6456 FR cells *in vivo* was assessed in an intracavitary peritoneal tumor model. Folate targeting resulted in a substantial increase of doxorubicin association to ascitic tumor cells (17-fold), lower ascitic fluid levels, and major reduction of doxorubicin plasma levels (14-fold) as compared with nontargeted PLD (Fig. 4). Doxorubicin fluorescence was strikingly increased in cells isolated from folate targeted PLD-injected mice as compared with nontargeted PLD-injected mice (Fig. 5). In addition, the diffuse cell fluorescence pattern and the depth color scanning analysis (Fig. 5D) clearly indicate the presence of abundant nuclear fluorescence of doxorubicin in J6456 tumor cells from animals treated with folate-targeted PLD.

Discussion

Folate targeting of liposomal drug carriers has been shown in a number of studies but has yet to be optimized for effective therapeutic applications (5, 22, 23). Here, we have investigated the uptake of folate targeted liposomal carriers in the J6456 lymphoma tumor model up-regulated for the folate receptor. Folate targeting significantly increased liposome binding to folate receptor-expressing J6456 lymphoma cells in a lipid dose-dependent fashion (Fig. 1). Different concentrations of folate in liposomes

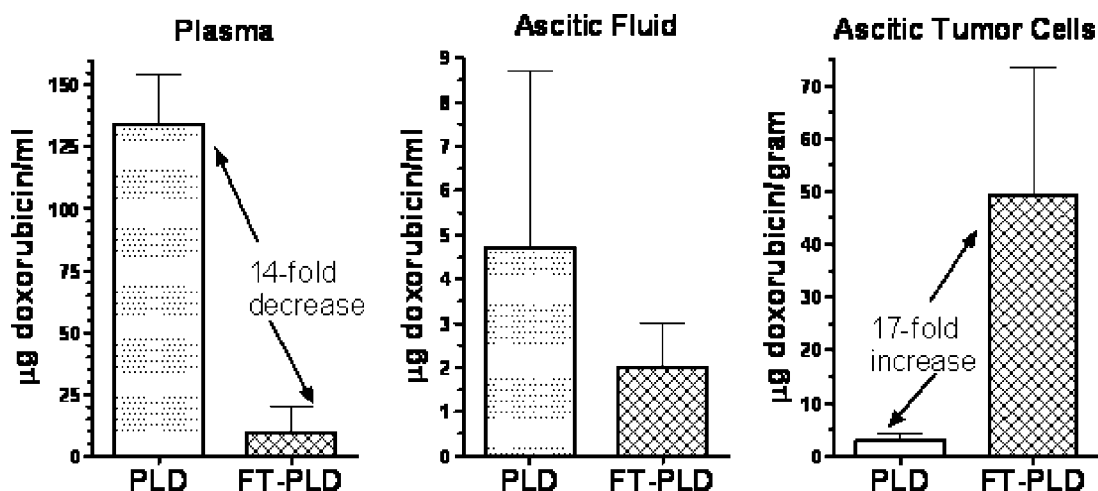
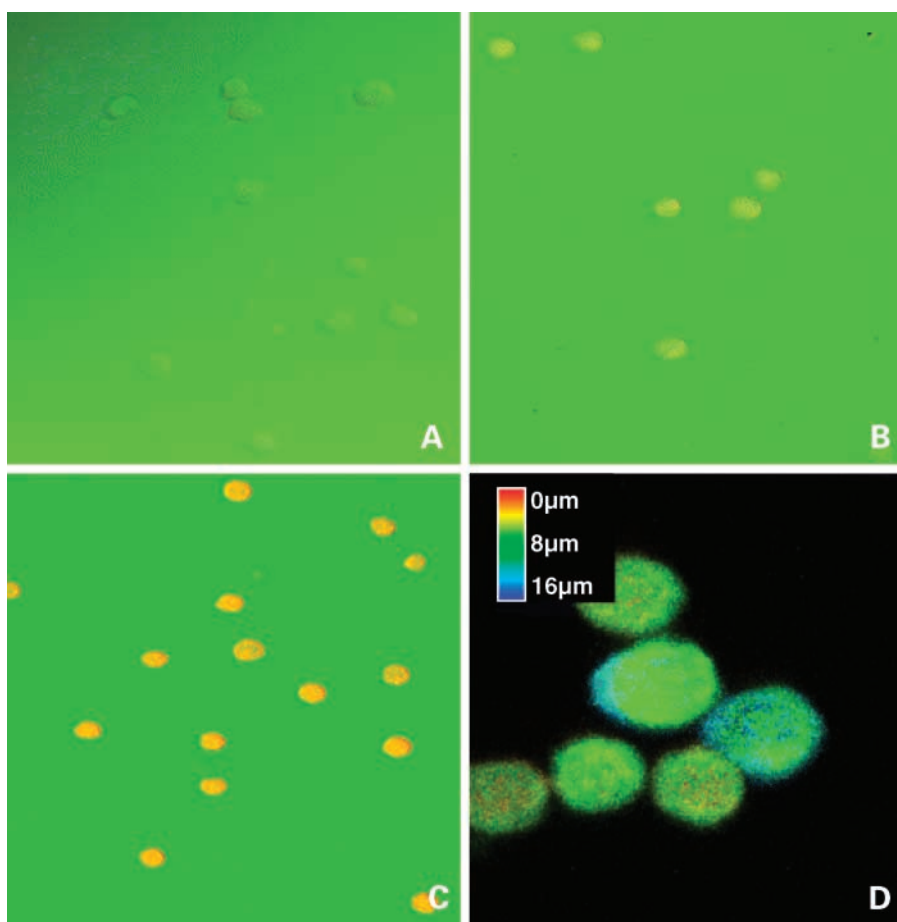


Figure 4. Doxorubicin levels in plasma and peritoneal compartments after i.p. injection of folate-targeted PLD. J6456-FR cells (10^6) were injected into the peritoneal cavities of BALB/c mice. After ascites developed, 10 mg/kg of PLD or FT-PLD (five mice each) were injected i.p. Sixteen hours later, mice were anesthetized, bled retroorbitally and sacrificed. The peritoneal cavity was rinsed with PBS and the ascitic exudate removed. Plasma, ascitic peritoneal fluid, and J6456-FR cellular doxorubicin levels were determined after extraction in acidic isopropanol and fluorescence was measured as described (21). Statistical analysis (unpaired *t* test) for PLD versus FT-PLD: plasma (left, $P < 0.0001$); ascitic fluid (middle, not significant); ascitic tumor cells (right, $P = 0.0029$).

Figure 5. Confocal analysis of ascitic J6456-FR cells from the peritoneal cavity of mice after i.p. injection of nontargeted PLD or folate-targeted PLD. Experimental conditions as for Fig. 3. Samples of ascitic cells were fixed for confocal analysis and mounted on glass slides for observation using a Zeiss Model 410 confocal microscope (488_{exc}, 520_{em}). **A**, control ascitic cells; **B**, nontargeted PLD-exposed ascitic cells; **C**, folate-targeted PLD-exposed ascitic cells; **D**, color-coded depth scan of folate-targeted PLD-exposed ascitic cells.



(ligand/phospholipid molar ratio), ranging between 0.03% and 0.5%, have been reported in the literature to be sufficient for liposome binding to folate receptor-bearing cells (10, 11, 18, 24, 25). In all these studies, the nature and concentration of the targeting ligand is an important variable in ligand-mediated cell association and uptake. The differences reported between the various ligands in optimal concentrations for receptor binding may be related to the accessibility of the folate moiety of the ligand, which, in turn, may be related to the PEG length (18), or to the PEG-folate chemical linkage (26). For instance, a linkage that enables folding-over of the folate ligand into the PEG chain may decrease accessibility or exposure of the folic acid to the receptor, thereby requiring a higher concentration of ligand for optimal binding.

To examine the cellular localization of the FTLs, we fluorescently labeled the liposome using water-soluble dextran-fluorescein. This marker was chosen after we noted in previous work with lipid-based fluorescent markers, such as rhodamine-DSPE and NBD-cholesterol, extensive exchange with cell membranes, particularly after long incubation times, overestimating liposome uptake.² In

addition, D-FITC is very hydrophilic and is practically unable to penetrate cells unless via liposome endocytosis. Impressive selective binding was achieved *in vitro* with D-FITC labeled FTL as seen in Figs. 2 and 3. After 3 hours of incubation, the liposomes were located in the periphery of the cells, mostly around the cell surface. After a 24-hour chase in liposome-free medium, there was evidence of liposome internalization in a significant number of cells, as indicated by depth scanning and a multifocal pattern of fluorescence, suggesting endocytic or other organelle compartment association.

In vivo binding of folate-targeted PLD is also followed by liposome internalization as seen in the confocal images (Fig. 5) of fluorescent doxorubicin in J6456-FR cells recovered from the ascites of i.p. injected mice. The increase in the degree of fluorescence in the cells from mice injected i.p. with FTLs compared with nontargeted PLD-injected mice was clearly noticeable by fluorescent microscopy and confirmed by direct fluorometric measurements of doxorubicin in cell extracts (Fig. 4). Whether this huge increase of internalized liposomal doxorubicin is released intracellularly in a form that is effective for cell killing remains to be shown in therapeutic studies. The distribution of fluorescence in Fig. 5C and D suggests nuclear localization of doxorubicin in tumor cells recovered

² H. Shmeeda and A. Gabizon, unpublished data.

from mice injected i.p. with folate-targeted PLD, indicating liposome internalization and drug release followed by rapid drug diffusion into the nucleus, in agreement with prior *in vitro* data with other cell lines (5, 14).

In addition, the i.p. injected FTLs reduced systemic levels of doxorubicin by 14-fold compared with nontargeted i.p. injected liposomes (Fig. 4). Such a marked drop in plasma levels will likely reduce systemic toxicity substantially. It also underscores the effect of a regional therapy approach with folate targeting because such a large shift in drug distribution is after all the result of a huge number of effective ligand-target interactions. Whereas the systemic use of FTL for cancer therapy remains a challenging approach given the need to maintain a long circulation time and to reach an extravascular target (5, 19), the potential therapeutic relevance of FTL for intracavitary or intravascular targets cannot be overemphasized. In fact, ovarian cancer, a tumor expressing folate receptor- α at a rate as high as 90% (1), may be a candidate for this approach given its predominant peritoneal surface spread. In addition, FTL may also be an attractive approach for the treatment of leukemic conditions, confined to the intravascular compartment and to the readily accessible bone marrow compartment, and often expressing folate receptor- β , as recently proposed by other investigators (12). The therapeutic efficacy of folate-targeted PLD or that of a similar folate-targeted liposomal cisplatin preparation (27) in appropriate folate receptor-expressing tumor models deserves investigation.

References

- Parker N, Turk MJ, Westrick E, Lewis JD, Low PS, Leamon CP. Folate receptor expression in carcinomas and normal tissues determined by a quantitative radioligand binding assay. *Anal Biochem* 2005;338:284–93.
- Elnakat H, Ratnam M. Distribution, functionality and gene regulation of folate receptor isoforms: implications in targeted therapy. *Adv Drug Deliv Rev* 2004;56:1067–84.
- Antony AC. Folate receptors: reflections on a personal odyssey and a perspective on unfolding truth. *Adv Drug Deliv Rev* 2004;56:1059–66.
- Low PS, Antony AC. Folate receptor-targeted drugs for cancer and inflammatory diseases. *Adv Drug Deliv Rev* 2004;56:1055–8.
- Gabizon A, Shmeeda H, Horowitz AT, Zalipsky S. Tumor cell targeting of liposome-entrapped drugs with phospholipid-anchored folic acid-PEG conjugates. *Adv Drug Deliv Rev* 2004;56:1177–92.
- Lee RJ, Low PS. Delivery of liposomes into cultured KB cells via folate receptor-mediated endocytosis. *J Biol Chem* 1994;269:3198–204.
- Shi G, Guo W, Stephenson SM, Lee RJ. Efficient intracellular drug and gene delivery using folate receptor-targeted pH-sensitive liposomes composed of cationic/anionic lipid combinations. *J Control Release* 2002;80:309–19.
- Zhou W, Yuan X, Wilson A, et al. Efficient intracellular delivery of oligonucleotides formulated in folate receptor-targeted lipid vesicles. *Bioconjug Chem* 2002;13:1220–5.
- Zhao XB, Lee RJ. Tumor-selective targeted delivery of genes and antisense oligodeoxyribonucleotides via the folate receptor. *Adv Drug Deliv Rev* 2004;56:1193–204.
- Reddy JA, Abburi C, Hofland H, et al. Folate-targeted, cationic liposome-mediated gene transfer into disseminated peritoneal tumors. *Gene Ther* 2002;9:1542–50.
- Saul JM, Annapragada A, Natarajan JV, Bellamkonda RV. Controlled targeting of liposomal doxorubicin via the folate receptor *in vitro*. *J Control Release* 2003;92:49–67.
- Pan XQ, Zheng X, Shi G, Wang H, Ratnam M, Lee RJ. Strategy for the treatment of acute myelogenous leukemia based on folate receptor β -targeted liposomal doxorubicin combined with receptor induction using all-trans retinoic acid. *Blood* 2002;100:594–602.
- Lee RJ, Low PS. Folate-mediated tumor cell targeting of liposome-entrapped doxorubicin *in vitro*. *Biochim Biophys Acta* 1995;1233:134–44.
- Goren D, Horowitz AT, Tzemach D, Tarshish M, Zalipsky S, Gabizon A. Nuclear delivery of doxorubicin via folate-targeted liposomes with bypass of multidrug-resistance efflux pump. *Clin Cancer Res* 2000;6:1949–57.
- Bathori G, Cervenak L, Karadi I. Caveolae—an alternative endocytotic pathway for targeted drug delivery. *Crit Rev Ther Drug Carrier Syst* 2004;21:67–95.
- Leamon CP, Low PS. Delivery of macromolecules into living cells: a method that exploits folate receptor endocytosis. *Proc Natl Acad Sci U S A* 1991;88:5572–6.
- Cabanes A, Tzemach D, Goren D, Horowitz AT, Gabizon A. Comparative study of the antitumor activity of free doxorubicin and polyethylene glycol-coated liposomal doxorubicin in a mouse lymphoma model. *Clin Cancer Res* 1998;4:499–505.
- Gabizon A, Horowitz A, Goren D, et al. Targeting folate receptor with folate linked to extremities of poly(ethylene glycol)-grafted liposomes: *in vitro* studies. *Bioconjug Chem* 1999;10:289–98.
- Gabizon A, Horowitz AT, Goren D, Tzemach D, Shmeeda H, Zalipsky S. *In vivo* fate of folate-targeted polyethylene-glycol liposomes in tumor-bearing mice. *Clin Cancer Res* 2003;9:6551–9.
- Barenholz Y, Amselem S. Quality control assays in the development and clinical use of liposome-based formulations. 2nd ed. Boca Raton (Florida): CRC Press; 1993.
- Gabizon AA. Selective tumor localization and improved therapeutic index of anthracyclines encapsulated in long-circulating liposomes. *Cancer Res* 1992;52:891–6.
- Stephenson SM, Low PS, Lee RJ. Folate receptor-mediated targeting of liposomal drugs to cancer cells. *Methods Enzymol* 2004;387:33–50.
- Reddy JA, Allagadda VM, Leamon CP. Targeting therapeutic and imaging agents to folate receptor positive tumors. *Curr Pharm Biotechnol* 2005;6:131–50.
- Pan XQ, Wang H, Lee RJ. Boron delivery to a murine lung carcinoma using folate receptor-targeted liposomes. *Anticancer Res* 2002;22:1629–33.
- Pan X, Wang H, Shukla S, et al. Boron-containing folate receptor-targeted liposomes as potential delivery agents for neutron capture therapy. *Bioconjug Chem* 2002;13:435–42.
- Leamon CP, Reddy JA. Folate-targeted chemotherapy. *Adv Drug Deliv Rev* 2004;56:1127–41.
- Gabizon A, Horowitz A, Tzemach D, Mak L, Zalipsky S, Abra R. Folate receptor targeting of pegylated (Stealth[®]) liposomal cisplatin enhances anti-tumor activity in mouse models without increasing toxicity. *Proc Am Assoc Cancer Res* 93rd Annual Meeting, Vol 43, 2002; Abstract 2063.

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