Antitumor Activity of Folate Receptor-Targeted Liposomal Doxorubicin in a KB Oral Carcinoma Murine Xenograft Model

Xing Q. Pan,[1] Huaqing Wang,[1] and Robert J. Lee[1,2]

Received September 30, 2002; accepted December 4, 2002

Purpose. The expression of folate receptor (FR) is amplified in many types of human cancers. Previously, FR-targeted liposomal doxorubicin (f-L-DOX) has been shown to exhibit superior and selective cytotoxicity against FR(+) tumor cells *in vitro* compared to nontargeted liposomal doxorubicin (L-DOX). This study further investigates f-L-DOX for its antitumor efficacy *in vivo* using a murine tumor xenograft model.

Methods. F-L-DOX composed of DSPC/cholesterol/PEG-DSPE/folate-PEG-DSPE (65:31:3.5:0.5, mole/mole) was prepared by polycarbonate membrane extrusion followed by remote loading of DOX. Athymic mice on a folate-free diet were engrafted with FR(+) KB cells. Two weeks later, these mice were treated with f-L-DOX, L-DOX, or free DOX in a series of six injections (given intraperitoneally on every fourth day at 10 mg/kg DOX) and monitored for tumor growth and animal survival. The plasma clearance profiles of the DOX formulations and the effect of dietary folate on plasma folate concentration were also analyzed.

Results. Plasma folate level remained in the physiologic range relative to that in humans. F-L-DOX exhibited an extended systemic circulation time similar to that of L-DOX. Mice that received f-L-DOX showed greater tumor growth inhibition and a 31% higher ($p < 0.01$) increase in lifespan compared to those that received L-DOX. Meanwhile, free DOX given at the same dose resulted in significant toxicity and was less effective in prolonging animal survival.

Conclusions. FR-targeted liposomes are a highly efficacious vehicle for *in vivo* delivery of anticancer agents and have potential application in the treatment of FR(+) solid tumors.

KEY WORDS: folate receptor; drug targeting; liposomes; doxorubicin; xenograft.

INTRODUCTION

Receptor-based tumor-selective delivery of therapeutic agents is a promising strategy for improving both the therapeutic efficacy and therapeutic index of cytotoxic drugs that exhibit dose-limiting toxicity. Targeted delivery has also been shown to bypass multidrug resistance in cultured tumor cells (1). The folate receptor (FR) is a 38-kDa glycosyl phosphatidylinositol (GPI)-anchored glycoprotein with highly restricted normal tissue expression. Meanwhile, FR is frequently elevated in many types of human cancers (2). There are two membrane-bound FR isoforms, α and β, with distinctive patterns of tissue distribution. Expression of FR-α is amplified in many epithelial-lineage tumors, including over 90% of ovarian carcinomas (2,3). In contrast, FR-β is frequently expressed in acute and chronic myelogenous leukemias (3,4). Selective targeting of FR(+) tumor cells can be achieved by covalently linking folic acid, a high-affinity ligand for both FR isoforms ($K_d \sim 0.1$ nM), to a drug or drug carrier (5,6). Folate conjugates have been shown to undergo FR-dependent cellular uptake and to be internalized by FR(+) cells via receptor-mediated endocytosis (5,6). This receptor-targeting strategy has been exploited in the delivery of imaging agents, anticancer agents, protein toxins, anti-T-cell antibodies, antisense oligodeoxyribonucleotides, gene transfer vectors, as well as liposomal drug carriers into receptor-positive cells (5,6).

To prepare FR-targeted liposomes, folate can be covalently attached via a polyethyleneglycol (PEG) linker to a phospholipid (7) or cholesterol anchor (8) and incorporated into the bilayer as a lipid ingredient during liposome preparation. Liposomal doxorubicin (L-DOX), which exhibits prolonged systemic circulation time and lower cardiotoxicity than free DOX, is currently in clinical use for the treatment of AIDS-related Kaposi's sarcoma (9). FR-targeted liposomal DOX (f-L-DOX) has shown superior cytotoxicity compared to L-DOX in cultured FR(+) tumor cells *in vitro* (10,11) and to overcome multidrug resistance in M103 murine carcinoma cells in an *in vivo* tumor adoptive assay (1). Furthermore, f-L-DOX has been shown to be more effective in prolonging the survival of mice carrying FR(+) murine lymphocytic L1210-JF cell ascites tumor (12). However, in solid tumor models, folate-coated liposomes showed only moderate to no significant enhancement in tumor localization (8,13,14), and their antitumor efficacy relative to nontargeted liposomes has not been reported in the literature.

In the present study, the antitumor activity of f-L-DOX is compared to that of L-DOX in an FR(+) murine xenograft tumor model. Data on tumor growth inhibition and animal survival are presented. Possible mechanisms of the observed enhancement in the antitumor activity of f-L-DOX and its potential applications in cancer therapy are also discussed.

METHODS

Materials

Folic acid, cholesterol (Chol), DOX, and Sepharose CL-4B chromatography resin were purchased from Sigma Chemical Co. (St. Louis, Missouri). Distearoylphosphatidylcholine (DSPC) and methoxypolyethyleneglycol (M.W. ~ 2000)-distearoylphosphatidylethanolamine (PEG-DSPE) were purchased from Avanti Polar Lipids (Alablaster, AL). Folate-polyethyleneglycol (M.W. ~ 3350)-distearoylphosphatidylethanolamine (f-PEG-DSPE) was synthesized as previously described (10). Polycarbonate membranes and Lipex™ lipid extruder were obtained from Northern Lipids, Inc. (Vancouver, Canada). Folate-free RPMI-1640 tissue culture media and newborn calf serum were purchased from Life Technologies (Rockville, Maryland).

Cell Culture

KB cells, derived from a human squamous cell carcinoma of the oral cavity, were obtained as a gift from Dr. Philip S. Low at Purdue University (West Lafayette, Indiana). The

[1] Division of Pharmaceutics, College of Pharmacy, The Ohio State University, Columbus, Ohio 43210.

[2] To whom correspondence should be addressed. (e-mail: lee.1339@osu.edu)

0724-8741/03/0300-0417/0 © 2003 Plenum Publishing Corporation

cells were maintained in folate-free RPMI 1640 medium supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% newborn calf serum, which provides the only source of folate. The cells were cultured as a monolayer in a humidified atmosphere containing 5% CO_2 at 37°C.

Liposome Preparation

Lipid compositions of DSPE/Chol/PEG-DSPE/f-PEG-DSPE (65:31:3.5:0.5, mole/mole) and DSPE/Chol/PEG-DSPE (65:31:4, mole/mole) were used for f-L-DOX and L-DOX, respectively. Liposomes were prepared by high-pressure polycarbonate membrane extrusion as described previously (12). Briefly, 30 mg of lipids of the above compositions was dissolved in chloroform, dried into a thin film under a stream of nitrogen, and then desiccated under vacuum for an additional 2 h. They were then hydrated in 5 mL of 400 mM sodium citrate, pH 4, with vortex mixing. The lipid suspension was subjected to six cycles of freezing and thawing and then extruded six times through a 100-nm pore-size polycarbonate membrane using a high-pressure Lipex extruder with a 10 mL barrel at 60°C and under 800 psi (generated by compressed nitrogen gas). The resulting small unilamellar vesicles were purified from nonentrapped molecules by size-exclusion chromatography on a Sepharose CL-4B column, preequilibrated in PBS (10 mM sodium phosphate, 150 mM NaCl, pH 7.4). The liposomes were eluted in the void volume fractions. Incorporation of DOX into the liposomes was performed by a remote-loading procedure based on the creation of a transmembrane pH gradient, as previously described (12). DOX concentrations in the liposomal samples were calculated from absorption at 480 nm following dissolution in 0.1% Triton X-100. Phospholipid concentration was determined by a colorimetic assay based on ammonium ferrothiocyanate chloroform phase extraction, as previously described (15). DOX loading efficiency obtained by this procedure is consistently found to be greater than 95% at the drug-to-lipid ratio of 1:10 (wt/wt) used in this study. The mean liposomal diameter and particle size distribution were measured by photon-correlation spectroscopy on a NICOMP 370 Submicron Particle Analyzer. The mean particle diameter was ~100 nm for all liposome preparations. Liposome samples were stored at 4°C and used within 4 weeks of preparation, during which period no significant (<1%) leakage of DOX was found.

Cellular Uptake of f-L-DOX Compared to L-DOX

KB cells grown as a monolayer were suspended by brief treatment with trypsin and then washed once with fresh culture medium. Aliquots of the KB cell suspension were incubated with f-L-DOX or L-DOX (containing 20μg/mL DOX) diluted in folate-free RPMI medium either with or without 1 mM free folic acid as an FR blocking agent for 1 h at 37°C. The cells were then washed three times with cold PBS and examined by flow cytometry using a Coulter Elite Flow Cytometer. Each analysis was generated using at least 10^5 cells.

Animal Tumor Model

Male athymic BALB/c (nu/nu) mice weighing 18–22 g were purchased from Charles River Laboratories (Wilmington, Massachusetts). The mice were maintained on a folate-free rodent diet (Cat. no. 117772, Dyets Inc., Bethlehem, Pennsylvania) on arrival and for the duration of the study. To generate tumor xenograft, 10^6 KB cells were injected subcutaneously (s.c.) using a 26-gauge needle in the left flank of the animals. The tumors reached palpable sizes of 10–20 mg at 14 days following tumor cell implantation. According to an earlier report from our lab (16), KB cells used in the engraftment had a maximum binding capacity (B_{max}) of ~10 pmol/mg cellular protein for [^{99m}Tc]HYNIC-folate.

Therapeutic Efficacy of f-L-DOX in Tumor-Bearing Mice

On day 1 (14 days after tumor cell implantation), the tumors reached sizes of 10–20 mm^3, at which time the mice (in groups of eight each) received one of the following treatments: (a) unloaded liposomes (with the same lipid composition as f-L-DOX), (b) f-L-DOX (10 mg/kg in DOX), (c) L-DOX (10 mg/kg in DOX), or (d) free DOX (10 mg/kg). The drug was given by i.p. injection on every fourth day (q4d) for six doses (days 1, 5, 9, 13, 17, and 21). Tumor size was measured on every fourth day, and animal survival was monitored daily. Tumor size was calculated using the equation: volume $= d_1 \times d_2^2 \times 0.6$. Survival data were presented in a Kaplan–Meier plot. In addition, treatment/control survival time ratio percentiles were calculated, as described previously (17). Statistical analyses of animal survival data were performed based on analysis of variance with a Bonferroni correction for multiple comparisons at the Ohio State University Center for Biostatistics.

Effect of Folate-Free Diet on Plasma Folate Concentration

Mice were first put on a regular rodent diet (Teklad 8640, Harlan, containing 3.19 mg/kg folate) for 2 weeks on arrival and then either continued on this diet or switched to a folate-free rodent diet (Cat. no. 117772, Dyets Inc.) for varying lengths of time. At the time of sacrifice, blood samples were collected by cardiac puncture. Red blood cells were removed by a 3-min centrifugation at 3000 *g*. Plasma samples were diluted to 1 mL with saline. Folate concentration was then determined immunologically by a competitive magnetic separation assay on a Bayer Immuno 1 System (Bayer Corporation, Tarrytown, New York) at the Clinical Lab at the Ohio State University Medical Center.

Plasma Clearance Rate of f-L-DOX, L-DOX, and Free DOX

Mice in groups of three animals each were treated with i.p. injection of 10 mg/kg of f-L-DOX, L-DOX, or free DOX. Blood samples (~100 μL) were collected from tail bleed at various time points. Plasma DOX concentration was determined based on extraction of DOX followed by fluorometry measurement, as described previously (18).

RESULTS

Uptake of f-L-DOX and L-DOX by Cultured KB Cells

Cellular uptake of liposomal DOX was assessed by flow cytometry based on DOX fluorescence. As shown in Fig. 1, uptake of f-L-DOX by the FR(+) KB cells was ~50 times greater than that of L-DOX. In addition, cellular uptake of

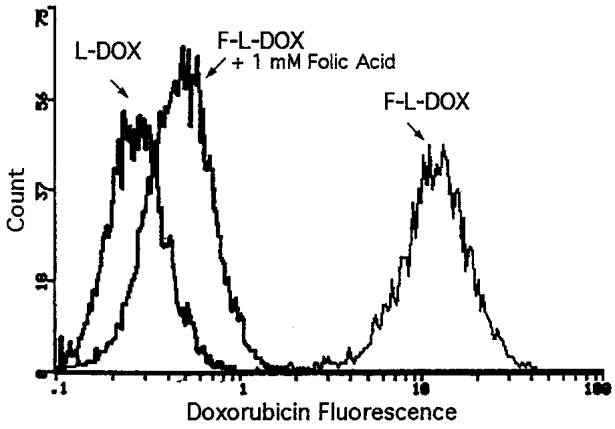

Fig. 1. Uptake of f-L-DOX and L-DOX by cultured KB cells. Liposomal uptake was determined based on DOX fluorescence by flow cytometry, as described in Methods.

f-L-DOX could be blocked by 1 mM free folic acid, indicating that the observed uptake was mediated by the FR (Fig. 1). These findings are consistent with those reported previously on the receptor-dependent cellular uptake of folate-coated liposomes (10,14).

Effect of Folate-Free Diet on Plasma Folate Concentration

Plasma folate concentrations following various amount of time on folate-free diet were determined by an automated immunoassay (see Methods). As shown in Fig. 2, in mice on a regular diet, plasma folate remained at a relatively high level (~175 nM). Meanwhile, mice that were switched to a folate-free diet showed significantly reduced plasma folate concentration (Fig. 2). Interestingly, however, even after 5 weeks on the folate-free diet, plasma folate concentration (~20 nM) remained in the physiologic range in humans, which, according to the standard target range used in the clinical laboratory, is between 14 and 51 nM. The plasma folate levels reported here are similar to those reported recently in another study on the effect of dietary folate on serum folate in mice (19).

Blood Clearance of f-L-DOX, L-DOX, and Free DOX

Although FR is absent in most normal tissues, it is conceivable that plasma proteins and/or normal tissues with low-affinity folate-binding activity might lead to altered biodistribution of folate-coated liposomes compared to that of nontargeted liposomes. The systemic clearance kinetics of f-L-DOX, L-DOX, and free DOX in mice was, therefore, determined and compared. Total plasma DOX concentrations following i.p. injection of the DOX formulations were measured by a fluorometric method (see Methods). As anticipated, both f-L-DOX and L-DOX showed a much greater systemic circulation time than free DOX, which showed a rapid clearance kinetics (Fig. 3). Mice treated with f-L-DOX showed only slightly lower plasma concentrations at all time points evaluated, indicating a similar clearance mechanism to that of L-DOX. These results are consistent with those in a previous report on the biodistribution of ^{111}In-labeled folate-coated liposomes (8).

Tumor Growth Inhibition by f-L-DOX, L-DOX, and Free DOX

The tumor inhibitory activities of unloaded liposomes, f-L-DOX, L-DOX, and free DOX were evaluated in athymic mice bearing KB xenograft tumor. As shown in Fig. 4, tumors rapidly increased in size when treated with unloaded liposomes. Mice that received six i.p. injections of 10 mg/kg of f-L-DOX showed complete suppression of tumor growth during treatment and for 2 weeks afterward, which was then followed by the eventual regrowth of the tumor (Fig. 4). In contrast, mice that received L-DOX showed only partial inhibition of tumor growth, followed by a more rapid regrowth following treatment cessation (Fig. 5). Meanwhile, mice that received the same dose of free DOX appeared to have died of

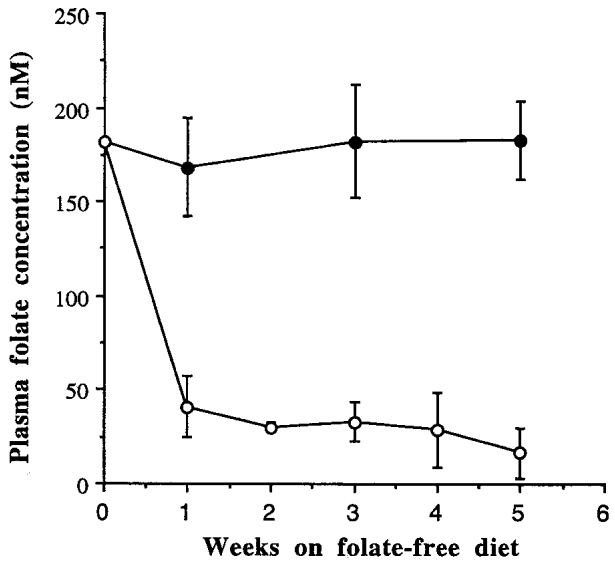

Fig. 2. Effect of dietary folate on plasma folate concentration in mice. Mice were kept either on a regular (●) or a folate-free (○) diet for varying lengths of time. Plasma folate was measured as described in Methods. The *error bar* is equal to 1 standard deviation ($n = 3$).

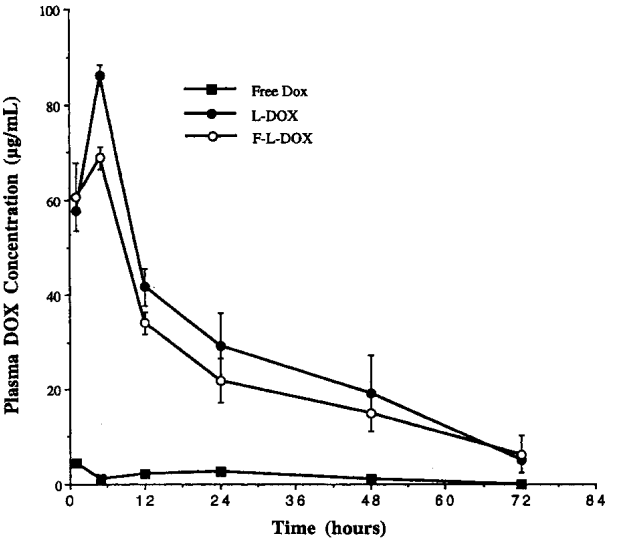

Fig. 3. Blood clearance profiles of f-L-DOX, L-DOX, and free DOX. Mice were treated with 10 mg/kg of f-L-DOX, L-DOX, or free DOX by i.p. injection. Plasma DOX concentrations at various time after injection were determined by a fluorometric assay, as described in Methods. *Error bar* shown is equal to 1 standard deviation ($n = 3$).

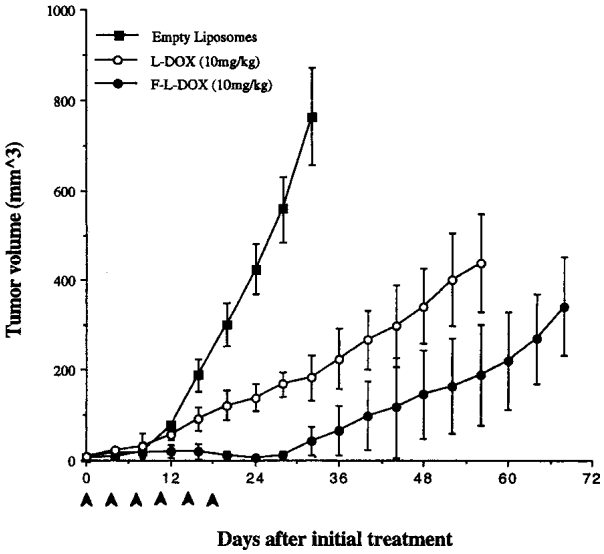

Fig. 4. Tumor growth inhibition by f-L-DOX or L-DOX. Nude mice with KB tumor xenograft were treated with a series of six i.p. injections (given on every fourth day, as indicated by the *arrows*) of liposomes containing 10 mg/kg DOX or with unloaded liposomes. Tumor size was measured for each animal on every fourth day starting from the day of the initial treatment. *Error bar* represents 1 standard deviation ($n = 8$).

toxicity by day 25 (Fig. 5). The above findings indicate that f-L-DOX is more effective than L-DOX in inhibiting tumor growth and that both f-L-DOX and L-DOX are less toxic to the animals than free DOX.

Effect of Treatment with f-L-DOX, L-DOX, and free DOX on the Survival of Tumor-Bearing Mice

Survival of mice carrying KB cell xenograft in response to the above treatment was determined as described in Methods. The results are presented in a Kaplan–Meier plot (Fig. 5), and the mean survival time and the percentile treatment/control ratio (T/C) for each treatment group are presented in Table I. The mice treated with f-L-DOX showed a 31% greater T/C value than those that received L-DOX ($p < 0.01$). Furthermore, both f-L-DOX and L-DOX were significantly more effective in prolonging mouse survival ($p < 0.01$) than free DOX. These findings indicate that f-L-DOX is therapeutically superior to L-DOX in this *in vivo* FR(+) tumor model.

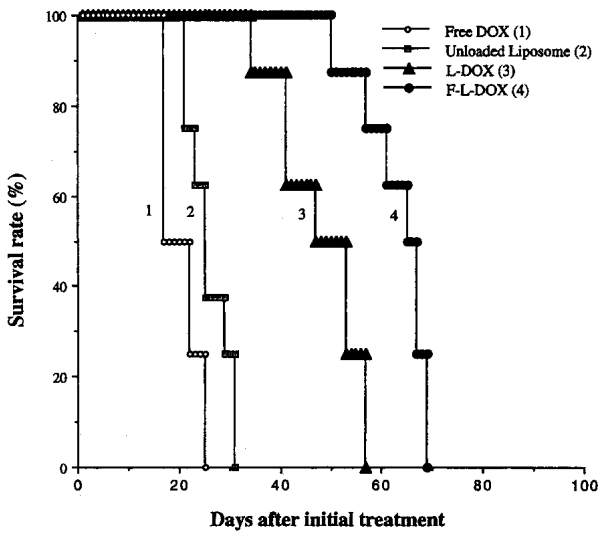

Fig. 5. Effect of f-L-DOX treatment on the survival of nude mice carrying KB tumor xenograft. The mice (eight mice in each group) were treated with unloaded liposomes, free DOX, L-DOX, or f-L-DOX via six i.p. injections (given on every fourth day) of liposomes containing 10 mg/kg DOX (except for the unloaded liposomes, which contain the same amount and composition of lipids as f-L-DOX), as described in Methods. Animal survival was recorded starting from the day of initial treatment (14 days after tumor cell implantation).

CONCLUSIONS

Results in this study have shown for the first time that FR-targeted liposomes can be used to enhance the therapeutic efficacy of a chemotherapy agent in an FR(+) solid tumor model without introducing excessive toxicity to the animal. Specifically, the FR-targeted f-L-DOX has been found to be significantly more effective in tumor growth inhibition and survival prolongation than the nontargeted L-DOX in an FR(+) murine tumor xenograft model.

Folic acid as a tumor-targeting ligand has several unique advantages, including (a) lack of immunogenicity, (b) small size, (c) chemical and functional stability, and (d) simple and defined conjugation chemistry (5). Previous studies evaluating folate-conjugated low-molecular-weight radiopharmaceuticals showed efficient FR-mediated uptake in kidneys and the tumor (16). Elevated uptake in the kidney might reflect receptor-mediated uptake by FR expressed on the apical side of the proximal tubules. Although this raises the concern of potential nephrotoxicity and significantly complicates the therapeutic application of low-molecular-weight folate conjugates, the biodistribution of FR-targeted liposomes is not likely to be affected by the presence of FR in the kidneys because their large size precludes glomerular filtration and, therefore, access to the receptor.

Plasma folate may interfere with FR binding and is, therefore, a potential factor for FR targeting *in vivo*. In addition, nutritional folate status has been shown to influence the efficacy and toxicity of chemotherapeutic agents (20). It is, therefore, important to establish that serum folate in the animal model used in this study corresponds to that in humans. Human serum folate, following recently FDA-mandated dietary supplementation of folic acid, is at ~42 nM (21). Earlier reports indicated that serum folate at this concentration should not significantly inhibit binding of FR-mediated liposomes, given their capacity for multivalent interaction with the cellular FR (7–9). Results in this study showed that mice were actually able to maintain a plasma folate level within the physiologic range of humans for at least 5 weeks on a folate-free diet (Fig. 2). In contrast, mice on a "regular" diet, which is fortified with 3.19 mg/kg folate, maintained a much higher level of serum folate, which was representative of the top few percent among the human population. The essentially "normal" levels of serum folate in mice on a folate-deficient diet are presumably a result of folate production by intestinal microflora because keeping rodents on a folate-deficient diet that also contains the antibiotic succinyl sulfathiazole has been reported to induce severe folate deficiency in rats (22). Results of this study, therefore, should be considered relevant to humans with respect to serum folate level.

Previous studies have shown that localization of lipo-

Table I. The Effect of f-L-DOX Treatment on the Survival of Mice with KB Cell Xenograft

Dosage form	Single injection dosage (mg/kg)	Total dosage (mg/kg)[a]	Survival time (days)[b]	Treatment/control (T/C, %)[c]
f-L-DOX	10	60	63 ± 9	242
L-DOX	10	60	48 ± 9	185
Free DOX	10	60	20 ± 4	77
Unloaded liposomes	0	0	26 ± 4	100

[a] The drug was injected i.p. on every fourth day for a total of 6 times.
[b] Mouse survival was recorded starting from the day of initial treatment (14 days after tumor cell implantation). Data are presented as mean ± standard deviation ($n = 8$).
[c] Survival time of mice treated with unloaded liposomes was defined as 100.

somes in solid tumor, even those with a targeting ligand, was primarity driven by a passive mechanism resulting from increased endothelial permeability and reduced lymphatic drainage, also known as the enhanced permeability and retention (EPR) effect (23). In previous reports, anti-HER2 immunoliposomes have shown very modest to no enhancement in tumor accumulation compared to the corresponding nontargeted liposomes (24). Similar observations of modest enhancement have been reported with FR-targeted liposomes and a bovine serum albumin–folate conjugate (58 kDa) (8,13,14,25). However, anti-HER2 immunoliposomes loaded with DOX have been shown to exhibit a significantly altered pattern of intratumoral distribution as well as improved therapeutic efficacy compared to nontargeted L-DOX (24). We believe a similar mechanism might be responsible for the enhanced therapeutic activity of FR-targeted liposomes. Because these liposomes have been shown to be efficiently internalized by cultured KB cells *in vitro* (7,10), it is conceivable that FR might facilitate cellular liposome uptake and internalization following extravasation of the liposomes.

Another factor affecting the intratumoral distribution and therapeutic efficacy of f-L-DOX is that DOX-induced necrosis and apoptosis within the tumor mass, which is enhanced by FR-dependent cellular uptake, might in turn facilitate the subsequent intratumoral diffusion of f-L-DOX. This is because reduction in viable tumor cells may partially remove the diffusion barrier that prevents efficient intratumoral distribution of the liposomes. In fact, a recent study showed that pretreatment of tumor histocultures with chemotherapeutic agents that induce tumor cell apoptosis resulted in greater permeability of the tumors to subsequently administered therapeutic agents (26).

The FR-mediated delivery pathway may also act by overcoming DOX efflux through membrane transporters related to multidrug resistance, as shown by Goren *et al.* (1). This would make FR-targeted liposomes a valuable delivery agent for the treatment of recurrent tumors with elevated P-glycoprotein expression.

F-L-DOX and L-DOX administered by i.p. injection showed similar plasma clearance profiles. This suggests that the presence of the folate ligand has only minimal effect on normal tissue and reticuloendothelial uptake of the liposomes. It is, therefore, possible that f-L-DOX and L-DOX will exhibit similar toxicity profiles including reduced cardiotoxicity compared to free DOX.

In summary, findings in this study suggest that injection of FR-targeted liposomes carrying an anticancer agent is a promising approach for enhancing their *in vivo* tumor inhibitory activity without introducing excessive normal tissue toxicity in FR(+) solid tumors. These findings are consistent with earlier results obtained in an FR(+) murine lymphocytic leukemia ascitic tumor model, which also showed a similar therapeutic advantage for f-L-DOX over nontargeted L-DOX in prolonging animal survival (12). Targeted delivery using folate-coated liposomes could potentially be used in the treatment of FR(+) cancer such as ovarian carcinomas, for which current therapy has limited effectiveness. Further preclinical studies in additional animal models are, therefore, warranted to better define the role of FR expression level, dietary folate, tumor model, and tumor size at the initiation of therapy on the antitumor activity and toxicity of f-L-DOX relative to L-DOX and to optimize the liposomal formulation and dosing regimen to achieve optimal tumor inhibition with these FR-targeted liposome. These efforts can potentially lead to the development of f-L-DOX as a clinical agent for the treatment of FR(+) tumors such as ovarian carcinomas.

ACKNOWLEDGMENTS

This work was supported in part by a grant (RPG-99-097-01-MGO) from the American Cancer Society and grant number 6113-02 from the Leukemia and Lymphoma Society.

REFERENCES

1. D. Goren, A. T. Horowitz, D. Tzemack, M. Tarshish, S. Zalipsky, and A. Gabizon. Nuclear delivery of doxorubicin via folate-targeted liposomes with bypass of multidrug-resistance efflux pump. *Clin. Cancer Res.* **6**:1949–1957 (2000).
2. P. Garin-Chesa, I. Campbell, P. E. Saigo, J. L. Lewis Jr., L. J. Old, and W. J. Rettig. Trophoblast and ovarian cancer antigen LK26. Sensitivity and specificity in immunopathology and molecular identification as a folate-binding protein. *Am. J. Pathol.* **142**:557–567 (1993).
3. J. F. Ross, P. K. Chaudhuri, and M. Ratnam. Differential regulation of folate receptor isoforms in normal and malignant tissues *in vivo* and in established cell lines—Physiologic and clinical implications. *Cancer* **73**:2432–2443 (1994).
4. J. F. Ross, H. Wang, F. G. Behm, P. Mathew, M. Wu, R. Booth, and M. Ratnam. Folate receptor type β is neutrophilic lineage marker and is differentially expressed in myeloid leukemia. *Cancer* **85**:348–357 (1999).
5. J. J. Sudimack and R. J. Lee. Targeted drug delivery via the folate receptor. *Adv. Drug Deliv. Rev.* **41**:147–162 (2000).
6. J. A. Reddy and P. S. Low. Folate-mediated targeting of therapeutic and imaging agents to cancers. *Crit. Rev. Ther. Drug Carrier Syst.* **15**:587–627 (1998).
7. R. J. Lee and P. S. Low. Delivery of liposomes into cultured KB cells via folate receptor-mediated endocytosis. *J. Biol. Chem.* **269**: 3198–3204 (1994).
8. W. Guo, T. Lee, J. J. Sudimack, and R. J. Lee. Receptor-specific

delivery of liposomes via folate-PEG-chol. *J. Liposome Res.* **10**: 179–195 (2000).
9. A. Gabizon. Pegylated liposomal doxorubicin: metamorphosis of an old drug into a new form of chemotherapy. *Cancer Invest.* **19**:424–436 (2001).
10. R. J. Lee and P. S. Low. Folate-mediated tumor cell targeting of liposome-entrapped doxorubicin *in vitro. Biochim. Biophys. Acta* **1233**:134–144 (1995).
11. A. Gabizon, A. T. Horowitz, D. Goren, D. Tzemach, F. Madelbaum-Shavit, M. M. Qazen, and S. Zalipsky. Targeting folate receptor with folate linked to extremities of poly(ethylene glycol)-grafted liposomes: *in vitro* studies. *Bioconjug. Chem.* **10**:289–298 (1999).
12. X. Q. Pan, X. Zheng, G. Shi, H. Wang, M. Ratnam, and R. J. Lee. 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* **100**: 594–602 (2002).
13. X. Q. Pan, H. Wang, and R. J. Lee. Boron delivery to a murine lung carcinoma using folate receptor-targeted liposomes. *Anticancer Res.* **22**:1629–1634 (2002).
14. M. J. Turk, D. J. Waters, G. Breur, W. R. Widmer, and P. S. Low. Folate-mediated targeting of liposomes to tumor cells and Mac-1 expressing cells *in vivo. J. Control. Release* **74**:369–376 (2001).
15. J. C. Stewart. Colorimetric determination of phospholipids with ammonium ferrothiocyanate. *Anal. Biochem.* **104**:10–14 (1980).
16. W. Guo, G. H. Hinkle, and R. J. Lee. ^{99m}Tc-HYNIC-folate, a novel receptor-based targeted radiopharmaceutical for tumor imaging. *J. Nucl. Med.* **40**:1563–1569 (1999).
17. J. Plowman, D. J. Dykes, M. Hollingshead, L. Simpson-Herren, and M. C. Alley. Human tumor xenograft models in NCI drug development. In B. Teicher (ed.), *Anticancer Drug Development Guide: Preclinical Screening, Clinical Trials, and Approval*, Humana Press, Totowa, New Jersey, 1997, pp. 101–125.
18. R. L. Hong, C. J. Huang, Y. L. Tseng, V. F. Pang, S. T. Chen, J. J. Liu, and F. H. Chang. Direct comparison of liposomal doxorubicin with or without polyethylene glycol coating in C-26 tumor-bearing mice: is surface coating with polyethylene glycol beneficial? *Clin. Cancer Res.* **5**:3645–3652 (1999).
19. J. Song, A. Medline, J. B. Mason, S. Gallinger, and Y.-I. Kim. Effects of dietary folate on intestinal tumorigenesis in the Apc Min mouse. *Cancer Res.* **60**:5424–5440 (2000).
20. R. F. Branda, E. Nigels, A. R. Lafayette, and M. Hacker. Nutritional folate status influences the efficacy and toxicity of chemotherapy in rats. *Blood* **92**:2471–2476 (1998).
21. J. M. Lawrence, D. B. Petitti, M. Watkins, and M. A. Umekubo. Trends in serum folate after food fortification. *Lancet* **354**:915–916 (1999).
22. R. L. Walzem and A. J. Clifford. Folate deficiency in rats fed diets containing free amino acids or intact proteins. *J. Nutr.* **118**: 1089–1096 (1988).
23. R. K. Jain. Delivery of molecular medicine to solid tumors:lessons from *in vivo* imaging of gene expression and function. *J. Control. Release* **73**:7–25 (2001).
24. J. W. Park, K. Hong, D. B. Kirpotin, D. Papahadjopoulos, and C. C. Benz. Immunoliposomes for cancer treatment. *Adv. Pharmacol.* **40**:399–435 (1997).
25. T. Shinoda, A. Takagi, A. Maeda, S. Kagatani, Y. Konno, and M. Hashida. *In vivo* fate of folate-BSA in non-tumor- and tumor-bearing mice. *J. Pharm. Sci.* **87**:1521–1526 (1998).
26. J. L.-S. Au, S. H. Jang, and M. G. Wientjes. Clinical aspects of drug delivery to tumors. *J. Control. Release* **78**:81–95 (2002).