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A folate receptor-targeted liposomal formulation for paclitaxel

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Abstract

A novel liposomal formulation of paclitaxel targeting the folate receptor (FR) was synthesized and characterized. This formulation was designed to overcome vehicle toxicity associated with the traditional Cremophor EL-based formulation and to provide the added advantages of prolonged systemic circulation time and selective targeting of the FR, which is frequently overexpressed on epithelial cancer cells. The formulation had the composition of dipalmitoyl phosphatidylcholine/dimyristoyl phosphatidylglycerol/monomethoxy-polyethylene glycol $(PEG)_{2000}$-distearoyl phosphatidylethanolamine/folate-PEG_{3350}-distearoyl phosphatidylethanolamine (DPPC/DMPG/mPEG-DSPE/folate-PEG-DSPE) at molar ratios of (85.5:9.5:4.5:0.5) and a drug-to-lipid molar ratio of 1:33. The liposomes were prepared by polycarbonate membrane extrusion. The mean particle size of the liposomes was 97.1 nm and remained stable for at least 72 h at 4 °C. FR-targeted liposomes of the same lipid composition entrapping calcein were shown to be efficiently taken up by KB oral carcinoma cells, which are highly FR+. FR-targeted liposomes containing paclitaxel showed 3.8-fold greater cytotoxicity compared to non-targeted control liposomes in KB cells. Plasma clearance profiles of paclitaxel in the liposomal formulations were then compared to paclitaxel in Cremophor EL formulation. The liposomal formulations showed much longer terminal half-lives (12.33 and 14.23 h for FR-targeted and non-targeted liposomes, respectively) than paclitaxel in Cremophor EL (1.78 h). In conclusion, the paclitaxel formulation described in this study has substantial stability and favorable pharmacokinetic properties. The FR-targeted paclitaxel formulation is potentially useful for treatment of FR+ tumors and warrants further investigation.

Keywords: Paclitaxel; Liposomes; Folate receptor; Targeted drug delivery; Cancer

1. Introduction

Paclitaxel is a microtubule stabilizing drug and a potent chemotherapeutic agent that has shown substantial clinical efficacy for ovarian, breast, colon, head and neck and non-small cell lung cancers (Rowinsky and Donehower, 1995; Spencer and Faulds, 1994). Due to its poor aqueous solubility, paclitaxel is currently formulated in a 1:1 mixture of Cremophor EL, a polyethoxylated castor oil, and ethanol (Henningsson et al., 2001; Szebeni et al., 1998). This formulation has limited stability on dilution and is associated with significant vehicle related toxicity in the clinic (Song et al., 1996; Rowinsky et al., 1993). There has been much interest in the development of novel formulations of paclitaxel to address the issues of stability and vehicle toxicity (Constantinides et al., 2004). Liposomal formulations of paclitaxel and its derivatives have been reported previously and have shown encouraging efficacy in preclinical studies (Sharma et al., 1993; Sody et al., 2003; Paola et al., 2000; Sharma and Straubinger, 1994).

Tumor cell targeting is a promising strategy for enhancing the therapeutic potential of chemotherapy agents. Folate receptor (FR)-α is a glycosyl phosphatidylinositol (GPI)-anchored membrane protein that is selectively overexpressed in over 90% of ovarian carcinomas (Wu et al., 1999; Elnakat and Rantnam, 2004; Weitman et al., 1992) and, to various extents, in other epithelial cancers. FR-β, an isoform of FR-α, is overexpressed in myelogenous leukemias (Ross et al., 1999; Pan et al., 2002). A number of FR-targeted therapeutic and imaging agents have been evaluated in preclinical studies, including liposomal agents, with promising results (Sudimack and Lee, 2000; Zhao and Lee, 2004; Lee and Low, 1995; Pan et

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al., 2002). Particularly interesting is the observation that FR-targeted liposomal delivery can reverse multidrug resistance to doxorubicin (Gabizon et al., 2004). Given the high level of FR expression in ovarian carcinomas and the effectiveness of paclitaxel as a first-line agent for this type of cancer, there is great rationale in development of an FR-targeted formulation for paclitaxel.

In this study, a lyophilized FR-targeted liposomal formulation of paclitaxel was prepared and characterized in terms of its stability, FR-dependent cytotoxicity and pharmacokinetic properties.

2. Materials and methods

2.1. Chemicals

Paclitaxel was purchased from Polymed Therapeutics Inc. (Houston, TX, USA), monomethoxy polyethylene glycol 2000-distearoyl phosphatidylethanolamine (mPEG-DSPE), dipalmitoyl phosphatidylcholine (DPPC), dimyristoyl phosphatidylglycerol (DMPG) and distearoyl phosphatidylethanolamine (DSPE) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Cremophor EL, polyoxyethylene bisamine (M.W. 3350, H_2N-PEG-NH_2), dicyclohexylcarbodiimide (DCC), disuccinimidyl suberate (DSS), *N*-hydroxysuccinimide (NHS), folic acid (FA) and triethylamine (TEA) were all purchased from Sigma Chemical Co. (St. Louis, MO, USA). All reagents and solvents were of analytical or HPLC grade and were used without further purification.

2.2. Synthesis of folate-PEG-DSPE

Folate-PEG-DSPE was synthesized as follows: first, to synthesize folate-PEG-amine, FA:H_2N-PEG-NH_2:DCC:NHS:TEA (1.2:1:1.5:1.5:5, molar ratio) were dissolved in anhydrous dimethyl sulfoxide (DMSO), respectively. FA, H_2N-PEG-NH_2, NHS, TEA and DCC were then sequentially added. The reaction mixture was stirred at 300 rpm in 10 mL round bottom flask overnight at room temperature in the dark. The mixture was centrifuged to remove by-product dicyclohexyl urea. The supernatant was diluted in three volumes of 50 mM sodium carbonate (Na_2CO_3) and passed through a PD-10 desalting column to remove low molecular weight (M.W.) by-products. Folate-PEG-amine (compound 1) with a yield of 41% was collected in the void volume and lyophilized. Second, to synthesize an electrophilically activated lipid anchor, DSPE dissolved in anhydrous chloroform ($CHCl_3$) was reacted with 1.2× of DSS in the presence of 5× of TEA overnight at room temperature to yield DSPE-CO-(CH_2)-CO-NHS (compound 2) with a yield of 44%. Finally, to synthesize folate-PEG-DSPE, compounds 1 and 2 were reacted in $CHCl_3$ overnight at room temperature. The solvent was then removed on a rotary evaporator and the product was suspended in 50 mM Na_2CO_3 to form micelles. These were then dialyzed against deionized water using a membrane with a molecular weight cut-off (MWCO) of 14,000 Da. The product was then lyophilized to yield a yellow dry powder with a yield of 72%. The purity of the product was confirmed by HPLC as described previously (Lee and Low, 1995; Gabizon et al., 1999).

2.3. Liposome preparation

Liposomes were prepared by polycarbonate membrane extrusion, as described previously (Paola et al., 2000). The lipid compositions of the FR-targeted liposomes and non-targeted liposomes were DPPC/DMPG/mPEG-DSPE/folate-PEG-DSPE at molar ratios of 85.5:9.5:4.5:0.5 and DPPC/DMPG/mPEG-DSPE at molar ratios of 85.5:9.5:5, respectively. Briefly, lipid ingredients and paclitaxel, at a drug-to-lipid molar ratio of 1:33, were dissolved in $CHCl_3$ and dried on a rotary evaporation in a round bottom flask at 40 °C and then under vacuum. The lipid film was then hydrated with 10% sucrose. The lipid suspension was extruded five times each through 0.2 μm and then 0.1 μm pore size polycarbonate membranes on a Lipex lipid extruder from Northern Lipids Inc. driven by high pressure nitrogen. Liposome size distribution was determined by dynamic light scattering on a NICOMP Submicron Particle Sizer Model 370 (NICOMP, Santa Barbara, CA, USA). Fluorescent liposomes were prepared by the same method except lipid was initially hydrated in 50 mM calcein. The liposomes were purified by size exclusion chromatography on a Sepharose CL-4B column equilibrated in phosphate buffered saline (PBS, 130 mM NaCl, 20 mM Na_2HPO_4, adjusted to pH 7.4). Paclitaxel concentration in the liposomal preparation was analyzed by reverse phase HPLC using an AllTech RP C_{18} column (4.1 mm × 300 mm, 10 μm particle size) and an isocratic program with a solvent system of methanol/water 70:30 (v/v) on a Beckman System Gold HPLC system with a UV detector at 227 nm (flow rate 1 mL/min).

To detect the presence of paclitaxel free drug, the liposome preparation was subjected to 15 min centrifugation at 10,000 × *g*, under which condition unincorporated drug would precipitate. By a second method, the formulation was passed through a 0.22 μm filter. The amount of free drug was determined by the change in paclitaxel concentration in the liposome preparation.

To determine the effect of freeze drying, the liposomes were lyophilized with the addition of 10% (w/v) of sucrose on a Labconco Lyphlok 12 using a program of 12 h at −44 °C for primary drying and 12 h at 20 °C for secondary drying. Following rehydration in deionized water, the properties of the lyophilized liposomes, including size distribution, free drug content and colloidal stability during storage were analyzed as described above.

2.4. Cell culture

KB, a human oral carcinoma cell line, which has amplified FR expression were cultured as a monolayer in folate-free RPMI 1640 media (Life Technologies Inc., Bethesda, MD, USA), supplemented with 50 μg/mL penicillin, 50 μg/mL streptomycin and 10% fetal bovine serum and maintained in a humidified atmosphere containing 5% CO_2 at 37 °C.

2.5. Uptake of FR-targeted fluorescent liposomes by KB cells

KB cells were suspended by a brief exposure to trypsin-EDTA (Invitrogen, Carlsbad, CA, USA) and rinsed with pH 3.5 saline (130 mM NaCl, 20 mM NaAc) and then PBS at 4 °C to remove receptor-bound free folate. For liposome uptake studies, the cells were incubated with FR-targeted or non-targeted control liposomes, each containing 150 nM calcein, in folate-free RPMI 1640 media for 60 min at 37 °C. The same experiment was repeated using media containing 1 mM free folic acid to determine the effect of FR blockade. At the end of incubation, the cells were washed three times with cold PBS and then visualized and photographed under a Nikon Eclipse 800 fluorescence microscope.

2.6. Cytotoxicity analyses

Cytotoxixity of folate liposomal paclitaxel was determined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as described previously (Stevens and Lee, 2003; Lee and Low, 1995) with minor modifications. KB cells were transferred to 96-well tissue culture plates at 5×10^4 cells per well 24 h prior to drug addition. The culture medium was then replaced with 200 μL of medium containing serial dilutions of paclitaxel formulations in FR-targeted liposomes, non-targeted control liposomes or Cremophor EL/ethanol. Following 2 h incubation at 37 °C, the cells were washed twice with PBS and cultured in fresh medium until untreated control wells reached >90% confluence. Then, 20 μL MTT stock solution (5 mg/mL) was added to each well, and the plate was incubated for 4 h at 37 °C. Medium was then removed and DMSO was added to dissolve the blue formazan crystal converted from MTT. Cell viability was assessed by absorbance at 570 nm measured on a Biorad microplate reader.

2.7. Pharmacokinetic studies

Plasma clearance kinetics of paclitaxel in the liposomal formulations and in Cremophor EL/ethanol was compared in ICR mice (Charles River Lab, Wilmington, MA, USA). Mice, in groups of three, received intravenous injections of paclitaxel in various formulations at 15 mg/kg body weight via tail vein (Sharma et al., 1994; Paola et al., 2000). Blood samples were collected in heparin-containing tubes at various time points (5, 30, 60, 240, 480 and 960 min). Plasma was isolated by centrifugation (10 min at 1500 × *g*), stored at −20 °C, and subsequently analyzed for paclitaxel by HPLC. During the analysis, the plasma samples were diluted to 0.5 mL with deionized water. Extraction of paclitaxel was accomplished by addition of 3 mL of *tert*-butyl methyl ether and vortex mixing for 30 s. The mixture was then centrifuged for 10 min at 1500 × *g* at 4 °C, after which 2.5 mL of the organic layer was transferred to a clean tube and evaporated to dryness by RapidVap Concentrator (LABCONCO, Kansas City, MI, USA). For HPLC sample loading, 100 μL of methanol was used to reconstitute the residue, and a 40 μL aliquot was injected onto a C18 reverse phase column for analysis, as described above. Pharmacokinetic parameters were determined using the Winnonlin software. Parameters, including area under the curve (AUC), mean residence time (MRT), total body clearance (CL) and plasma half-life for the distribution and elimination phase were determined.

3. Results

3.1. Synthesis and characterization of folate-liposomal paclitaxel

Folate-PEG-DSPE (Fig. 1) was synthesized as described in Section 2. FR-targeted liposomal formulation of paclitaxel was prepared by polycarbonate membrane extrusion. Paclitaxel incorporation efficiency, determined by subtracting free drug fraction, as described in Section 2, was found to be 97.6%. The mean diameter of a typical liposome preparation was 93.4 nm with narrow size distribution. Lyophilization of the liposomal formulations in the presence of sucrose, as a lyoprotectant, only resulted in a small increase in particle size (93.4–97.1 nm) upon rehydration.

The stability of the liposomal formulations of paclitaxel was monitored by changes in particle size and drug retention over a 72 h period during storage at 4 °C following rehydration of the lyophilized formulation. As shown in Figs. 2 and 3, the liposomal formulation showed excellent colloidal stability and drug retention during this period. There were no visible changes to the physical appearance of the formulation or signs of drug precipitation.

3.2. Cellular uptake and cytotoxicity of folate-PEG-liposomes

Uptake of folate-liposomes of the same composition, entrapping a fluorescent dye calcein, was analyzed in FR+ KB cells. As shown in Fig. 4, FR-targeted liposomes showed much greater cellular uptake and liposome internalization compared to non-

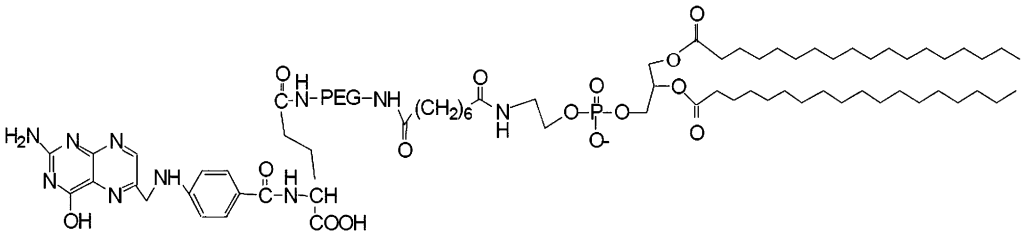

Fig. 1. Structure of folate-PEG-DSPE.

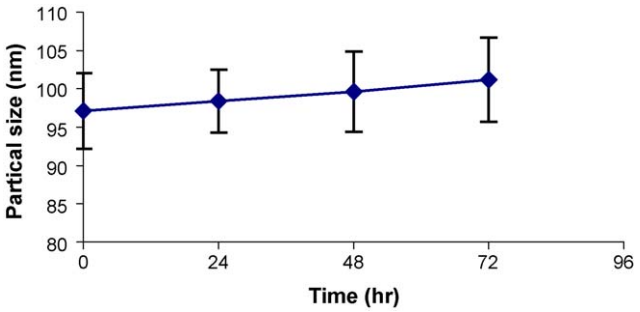

Fig. 2. Stability of FR-targeted liposomal paclitaxel as measured by the particle size ($n = 5$). Liposomes were stored at 4 °C prior to analysis.

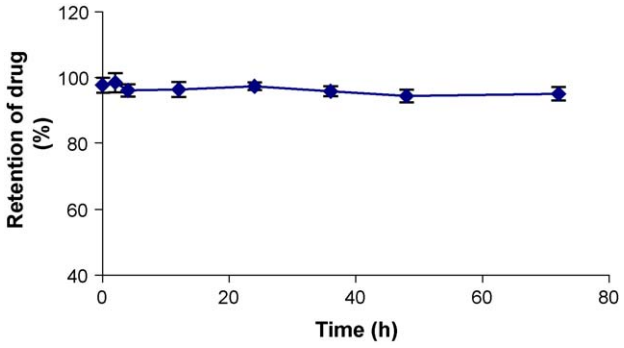

Fig. 3. Paclitaxel encapsulation and stability of FR-targeted liposomes as measured by percentage of paclitaxel remaining in the liposomes ($n = 5$). Liposomes were stored at 4 °C prior to analysis.

targeted control liposomes and the uptake was blocked by 1 mM free folate. These results indicated that these liposomes have efficient interactions with the cellular folate receptor.

Cytotoxicity of FR-targeted and non-targeted control paclitaxel liposomes, and paclitaxel in Cremophor EL/ethanol, was determined in FR+ KB cells using an MTT assay. The results are summarized in Table 1. The results showed that FR-targeted liposomal paclitaxel had approximately four times lower IC_{50} value compared to that of non-targeted liposomal paclitaxel and 2.5 times lower than that of paclitaxel in Cremophor EL/ethanol. The differential cytotoxicity was eliminated in the presence of 1 mM free folate. These data suggested FR-dependence of the cytotoxicity exhibited by the FR-targeted liposomal paclitaxel.

Table 1
Cytotoxicity of paclitaxel liposomes in KB cells determined by MTT assay

	IC_{50} (μM) ($n = 3$)
Paclitaxel in FR-targeted liposomes	0.048 ± 0.015
Paclitaxel in FR-targeted liposomes + 1 mM free folic acid	0.151 ± 0.052
Paclitaxel in non-targeted liposomes	0.184 ± 0.063
Paclitaxel in Cremophor EL/ethanol	0.121 ± 0.044

IC_{50}, paclitaxel concentration resulting in 50% cell killing.

3.3. Pharmacokinetic properties of FR-targeted liposomal paclitaxel

Plasma clearance kinetics of the liposomal formulations of paclitaxel was compared to that of Cremophor EL/ethanol formulation in ICR mice. As shown in Fig. 5, all three formulations showed biphasic clearance kinetics. The liposomal formulations exhibited much longer plasma half-lives compared to the Cremophor EL formulation. FR-targeted liposomes were cleared faster than the non-targeted control formulation. Pharmacokinetic parameters were derived from data analysis using Winnonlin. As shown in Table 2, the clearance of paclitaxel in Cremophor EL was 30 or 16.5 times greater than that of non-targeted liposomes and FR-targeted liposomes, respectively. Therefore, the longer half-lives of the liposomal formulation were likely the result of reduced plasma clearance.

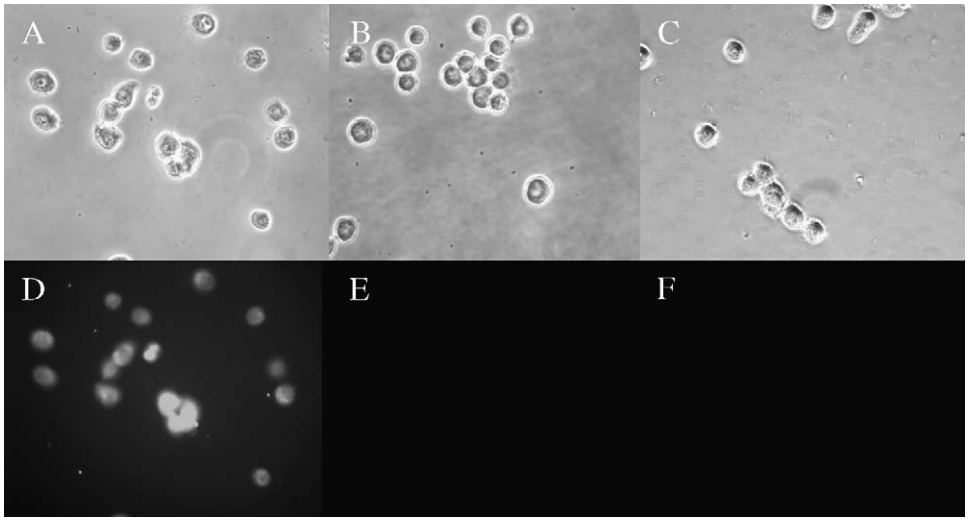

Fig. 4. Uptake of FR-targeted liposomal calcein and non-targeted liposomal calcein by KB cells. KB cells were treated with FR-targeted liposomal calcein, non-targeted liposomal calcein or FR-targeted liposomal calcein containing 1 mM of free folic acid and photographed in both the fluorescence (dark fields) and phase-contrast modes (bright fields) on a microscope as described in Section 2. (A) and (D) Cells treated with FR-targeted liposomal calcein; (B) and (E) cells treated with non-targeted liposomal calcein and (C) and (F) cells treated with FR-targeted liposomal calcein containing 1mM free folic acid. (A–C) Are micrographs taken in the phase-contrast mode and (D–F) are the same fields viewed in the fluorescence mode.

Table 2
Pharmacokinetic parameters of pacliaxel formulations in mice following i.v. bolus administration

Formulation	AUC (mg mL^{-1} h)	MRT (h)	$t_{1/2\alpha}$ (h)	$t_{1/2\beta}$ (h)	CL (mL h^{-1})
Cremophor EL/ethanol	0.06 ± 0.001	2.15 ± 0.06	0.115 ± 0.003	1.78 ± 0.153	12.41 ± 0.288
Non-targeted liposomes	0.730 ± 0.160	19.78 ± 6.39	0.404 ± 0.085	14.23 ± 4.60	0.41 ± 0.009
FR-targeted liposomes	0.460 ± 0.093	16.76 ± 5.99	0.288 ± 0.041	12.33 ± 4.35	0.75 ± 0.175

The values are means of three experiments (n = 3–5 animals per group).

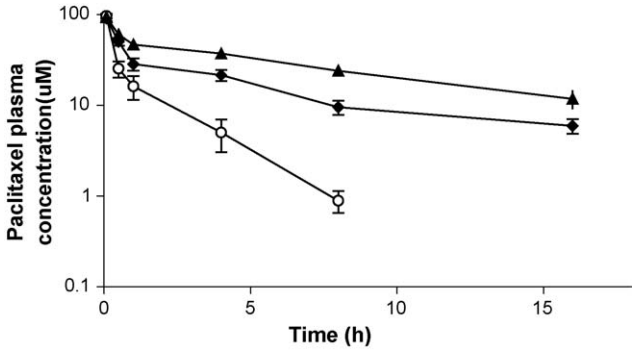

Fig. 5. Plasma concentration vs. time curves for paclitaxel formulations in mice. Paclitaxel formulations were administered via i.v. tail vein injection at a dose of 15 mg/kg paclitaxel. The formulations used were Cremophor EL/ethanol (◯), FR-targeted paclitaxel liposomes (●) and non-targeted paclitaxel liposomes (▲). Each data point was the average of three to five animals and error bar equaled one standard deviation.

4. Discussion

We described in this report the synthesis and characterization of a novel FR-targeted liposomal formulation for paclitaxel. Paclitaxel has very poor water solubility and limited lipid solubility. Taxol, which is based on Cremophor EL/ethanol and is in current clinical use, has significant vehicle related hypersensitivity reaction, which necessitates premedication with steroids and antihistamine. Previous attempts on developing liposomal formulations of paclitaxel have yielded encouraging results, although some formulations had shown limited stability. The formulation described in this study was composed of DPPC/DMPG/mPEG-DSPE/folate-PEG-DSPE. The rationale for this design was that paclitaxel has a strong propensity for crystallization in an aqueous medium and cannot efficiently incorporate into a lipid bilayer (Sharma and Straubinger, 1994; Corinna et al., 1998; Liversidge et al., 2003). However, phospholipids are excellent surfactants, which can provide colloidal stabilization of paclitaxel nanocrystals. Negatively charged lipids DMPG and mPEG-DSPE can theoretically provide electrostatic and steric stabilization of the liposomal formulation. The incorporation of mPEG-DSPE can also extend the plasma circulation half-life of the liposomal formulation by shielding the particle surface against opsonization and reducing uptake by the reticuloendothelial system (Klibanov et al., 1990; Allen, 1994; Lasic, 1997). Data in this report showed that stable liposomal particles with narrow size distribution and high drug incorporation can be prepared using this lipid composition. Furthermore, the formulation can be lyophilized and has exhibited substantial colloidal stability during storage following rehydration.

FR is a tumor marker that is consistently overexpressed in ovarian carcinomas. Results in this study are consistent with previous reports on FR-targeted liposomes (Stevens and Lee, 2003; Stevens et al., 2004). The synthesis of folate-PEG-DSPE described utilized a new linker between the PEG and the lipid anchor, which did not affect FR-targeting properties as shown by the KB cell uptake studies using fluorescent liposomes. The enhancement in cytotoxicity exhibited by FR-targeted liposomal paclitaxel was about four-fold over the non-targeted control. The lack of a greater targeting ratio might be attributed to the gradual release of paclitaxel into the media and its non-specific uptake by tumor cells during the 1 h incubation period. The results nonetheless demonstrated a FR-dependence of the cytotoxicity. Since free paclitaxel is more rapidly cleared in vivo, this factor might have a lesser effect on tumor cell targeting of the liposomes in vivo.

Pharmacokinetic studies on the liposomal formulations of paclitaxel indicated a longer systemic circulation time relative to that of the Cremophor EL formulation. This result suggested greater in vivo stability of the formulation following i.v. administration compared to Cremophor EL micelles. The AUCs of the liposomal formulations were much greater than the Cremophore EL formulation. Given the size of these liposomes and the long circulation time, it is likely that these will preferentially accumulate in solid tumors based on the enhanced permeability and retention (EPR) effect via a passive targeting mechanism. The FR-targeted formulation had faster clearance than the non-targeted formulation. This was consistent with previous studies on FR-targeted liposomes, which showed that folate-liposomes were preferentially taken up by the liver and spleen of rats and that the increased uptake was greater in rats on a folate free diet (Gabizon et al., 2004). However, the same studies showed comparable tumor uptake of the FR-targeted and non-targeted control liposomes in solid tumors. Targeting solid tumors in vivo is challenging due to the rate-limiting step of extravasation and the limited potential for liposomes to diffuse within a solid tumor based on their size. Another complicating factor is that tumor infiltrating macrophages are likely FR positive. In fact, Turk et al. (2004) showed that FR-targeted liposomes preferentially target macrophages even in the presence of FR+ tumor cells. This raises the possibility that FR+ tumor infiltrating macrophages may constitute the actual target cell population in solid tumors.

5. Conclusion

A novel FR-targeted liposomal paclitaxel formulation is reported. This formulation has good drug loading properties and exhibit excellent colloidal stability. Furthermore, the formula-

tion was efficiently taken up by FR+ KB cells and exhibit FR-dependent cytotoxicity in these cells. Pharmacokinetic studies indicated prolonged circulation time of the liposomal paclitaxel relative to the Cremophor EL formulation. Further studies are warranted to investigate the potential therapeutic advantage of the FR-targeted liposomal formulation and possible mechanism of in vivo tumor targeting.

Acknowledgments

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