A Folate Receptor–Targeted Lipid Nanoparticle Formulation for a Lipophilic Paclitaxel Prodrug

Phillip J. Stevens,[1] Masaru Sekido,[1] and Robert J. Lee[1,2]

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Purpose. The anticancer drug paclitaxel has poor aqueous solubility and is difficult to formulate in a lipid-based formulation due to its limited lipid solubility. Paclitaxel-7-carbonyl-cholesterol (Tax-Chol), a prodrug of paclitaxel with increased lipophilicity, was therefore synthesized and evaluated for incorporation into a lipid nanoparticle (LN) formulation, which also contained folate-polyethylene glycol-cholesterol (f-PEG-Chol) as a ligand that targets the tumor marker folate receptor (FR). This novel formulation was designed for prolonged systemic circulation and selective targeting of tumor cells with amplified FR expression.

Methods. Tax-Chol was synthesized. FR-targeted LNs, composed of distearoyl phosphatidylcholine (DSPC)/triolein/Chol oleate/PEG-Chol/f-PEG-Chol (40:40:18:2.0:0.5, mole/mole), were then prepared by solvent dilution followed by diafiltration. FR-targeted LNs containing Tax-Chol were then evaluated for cytotoxicity in KB, a human oral carcinoma cell line, and M109, a murine lung carcinoma cell line, both of which are FR(+) and in FR(−) Chinese hamster ovary (CHO) cells. Furthermore, tumor growth inhibition and animal survival in response to treatment with FR-targeted LNs and control formulations were evaluated in BALB/c mice bearing subcutaneously engrafted M109 tumors.

Results. The LNs had a mean diameter of 130 nm and Tax-Chol incoporation efficiency of greater than 90% and exhibited excellent colloidal stability. FR-targeted LNs showed greater uptake and cytotoxicity in FR(+) KB and M109 cells than nontargeted LNs. Furthermore, treatment of mice bearing M109 tumors with FR-targeted LNs resulted in significantly greater tumor growth inhibition and animal survival compared to treatment with nontargeted LNs or paclitaxel formulated in Cremophor EL.

Conclusion. FR-targeted LNs containing Tax-Chol are a promising novel formulation for the treatment of FR(+) tumors and further preclinical studies are warranted.

KEY WORDS: folate receptor; lipid nanoparticles; lipophilic prodrug; paclitaxel; tumor targeting.

INTRODUCTION

Paclitaxel is an antimicrotubule agent used clinically for the treatment of ovarian, breast, and non-small cell lung carcinomas, as well as AIDS-related Kaposi's sarcoma (1). Due to poor aqueous solubility, clinical paclitaxel is currently formulated in a 1:1 mixture of Cremophor EL and ethanol (Taxol), which is further diluted in saline or dextrose prior to i.v. infusion. The diluted formulation is unstable and frequently produces drug precipitation, which necessitates filtration. Furthermore, Cremophor EL has been reported to elicit numerous vehicle-related side effects, including hypersensitivity and nephrotoxicity (2). Development of a formulation of paclitaxel with increased stability and reduced toxicity is therefore desirable. To this end, paclitaxel has been incorporated into the lipid phase of liposome or lipid nanoparticle formulations, which exhibited reduced toxicity and enhanced antitumor efficacy in animal models (3–6). However, these formulations released paclitaxel in storage, which then precipitated, thus lacking the necessary long-term stability. The instability of these liposomal and lipid nanoparticle formulations was possibly due to the limited lipid-solubility of paclitaxel. The limited solubility of paclitaxel in both the aqueous and the lipid phases resulted in its strong propensity for precipitation. A potential strategy to overcome this obstacle is to synthesize a paclitaxel prodrug with an altered solubility profile. Previous efforts on developing paclitaxel prodrugs have mostly been focused on derivatives with increased aqueous solubility (7–16). However, in order to improve incorporation in lipidic carriers, a prodrug with increase lipophilicity would be required. In this study, a paclitaxel-cholesterol prodrug was synthesized and incorporated in to lipid nanoparticles (LNs). In addition, to increase tumor-selective cytotoxicity, these LNs were targeted to the folate receptor (FR), which is overexpressed in a wide variety of human tumors, including greater than 90% of ovarian carcinomas (17,18). The stability, *in vitro* cytotoxicity, and *in vivo* antitumor activity of these LNs were characterized.

MATERIALS AND METHODS

Materials

Paclitaxel was purchased from Polymed Therapeutics, Inc. (Houston, TX, USA), triolein from Chem. Service (West Chester, PA, USA), distearoyl phosphatidylcholine (DSPC) and polyethylene glycol 2,000-distearoyl phosphatidylethanolamine (PEG-DSPE) from Avanti Polar Lipids (Alabaster, AL, USA), and cholesterol oleate (Chol oleate) and cholesteryl chloroformate from Sigma Chemical (St Louis, MO, USA). $PEG_{2,000}$-Cholesterol (PEG-Chol) and folate-$PEG_{3,350}$-cholesterol (f-PEG-Chol) were synthesized as described previously (19).

Synthesis of Paclitaxel-2′-carbonyl-Cholesterol (Tax-Chol)

The synthetic method is summarized in Fig. 1. First, 100 mg paclitaxel dissolved in 5 ml of chloroform was combined with 1.5 molar excess of cholesteryl chloroformate in 5 ml of chloroform, 10 ml of *N,N*-diisopropylethylamine, and 5 ml acetonitrile. The mixture was stirred overnight at ambient temperature and then dried on a rotary evaporator. The resulting off-white precipitate was then dissolved in ethyl acetate/hexane (3:1) and extracted with water, dried, and then redissolved in chloroform. The formation of the product was confirmed by thin-layer chromatography using ethyl acetate/hexane (3:1) as the mobile phase (R_f of paclitaxel = 0.4, R_f of Tax-Chol = 0.92, data not shown). Further purification of the product was then carried out on a silica gel column using ethyl acetate/hexane (3:1) as the mobile phase. The product, Tax-Chol, was identified by mass spectrometry performed on a Micromass Q-TOF II mass spectrometer (Waters, Milford, MA) equipped with an orthogonal electrospray operated in

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

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

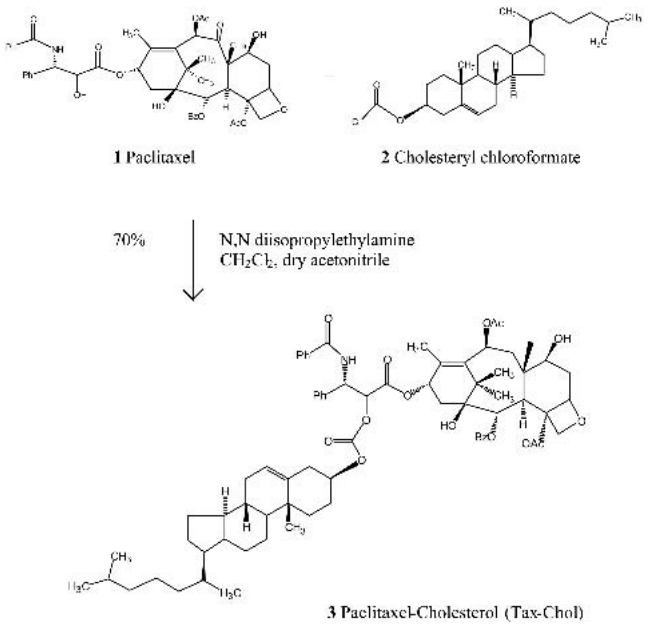

Fig. 1. Synthesis of paclitaxel-7-carbonyl-cholesterol (Tax-Chol).

positive ion mode [yield: 70%, mass spectrum (M+Na) ($C_{75}H_{95}NO_{16}Na$), calculated: 1288.6549; found: 1288.6581]. HPLC (High Performance Liquid Chromatography) analysis was performed using an Altech Econsil C_{18} column (150 mm × 3.2 mm, 5 μm) (Alltech, Deerfield, IL) on a Spectraphysics ISOChrom LC Pump, and a Spectra 100 variable wavelength detector (229 nm for both paclitaxel and Tax-Chol), and an HP3392A integrator with methanol/water (80:20) as the mobile phase at a flow rate of 0.8 ml/min, yielding retention times of 7.5 min for Tax-Chol and 6 min for paclitaxel, respectively.

Preparation of Tax-Chol Lipid Nanoparticles

LN formulations incorporating Tax-Chol were prepared by solvent dilution followed by diafiltration. The FR-targeted and nontargeted LNs had compositions of DSPC/triolein/Chol oleate/PEG-Chol/f-PEG-Chol (40:40:18:2.0:0.5), and DSPC/triolein/Chol oleate/PEG-Chol (40:40:18:2.5), respectively. For LN preparation, the components were dissolved in ethanol/*tert*-butanol (1:1, v/v), at a concentration of 10 mg/ml, and then diluted 1:10 into a stirred solution (1200 rpm) of 0.9% NaCl. Both the aqueous medium and the lipid solution were prewarmed to and kept at 40°C during the mixing. The LN preparation was then concentrated, washed, and buffer exchanged by ultrafiltration/diafiltration against 0.9% NaCl using a Spectropor MiniKros tangential flow system and a hollow-fiber cartridge with a molecular weight cutoff of 400 kDa (Spectrum Labs, Ranch Dominguez, CA). The final LN preparation had a lipid concentration of 60 mg/ml and a Tax-Chol concentration of 3 mg/ml. LN size distribution was determined by dynamic light scattering on a NICOMP Submicron Particle Sizer Model 370 (Nicomp, Santa Barbara, CA) at the time of liposome preparation and after storage for varying time at 4°C or 25°C.

Cell Culture

KB, a human oral carcinoma cell line and M109, a murine lung carcinoma cell line, both of which are (FR), were kindly provided by Dr. Philip Low at Department of Chemistry, Purdue University (West Lafayette, IN, USA). Chinese hamster ovary (CHO) cells, which are FR(−), were obtained from Dr. Manohar Ratnam at the Medical College of Ohio (Toledo, OH, USA). The KB and M109 cells were cultured as monolayers in folate-free RPMI 1640 media supplemented with penicillin, streptomycin, and 10% fetal bovine serum and propagated in a humidified atmosphere containing 5% CO_2 at 37°C. CHO cells were cultured as monolayers in RPMI 1640 media supplemented with penicillin, streptomycin, and 10% fetal bovine serum.

Cytotoxicity Analyses

Cytotoxicity of LNs was determined by an MTT assay as described previously (20). The assay determines cell viability based on the mitochondrial conversion of a water-soluble tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolum bromide; MTT] to the water-insoluble blue formazan product. Cells were transferred to 96-well tissue culture plates at 2×10^4 cells per well 24 h prior to drug treatment. The culture medium was then replaced with 200 μl of medium containing serial dilutions of LNs or paclitaxel formulated in a 1:1 mixture of Cremophor EL, with 2% bovine serum albumin (BSA) as a blocking agent. Following 2 h incubation under 5% CO_2 at 37°C, the cells were washed twice with PBS and cultured in fresh medium until untreated control wells reached >90% confluence (~72 h). Twenty microliters of MTT stock solution (5 mg/ml in PBS) was then added to each well, and the plate was incubated for 4 h at 37°C. Medium was then removed, and cells were solubilized in 200 μl isopropanol containing 0.1 M HCl. Cell viability was assessed by absorbance at 570 nm measured on an automated plate reader (Biorad, Hercules, CA). To determine FR selectivity, cytotoxicity assay was repeated using serial dilutions containing 1 mM free folic acid to block FR binding.

In Vitro Drug Release

The release of the prodrug Tax-Chol or paclitaxel in the same LN formulation, and paclitaxel in Cremophor EL, in the presence of 45 mg/ml BSA, was monitored by a dialysis method, as described previously (6). The dialysis was carried out at 37°C using Spectra/Por dialysis membranes with molecular weight cutoff of 14 kDa and phosphate-buffered saline (pH 7.4) as the sink solution (6). The initial concentration of Tax-Chol and paclitaxel in the formulations was 3 mg/ml. The concentration of drug was analyzed at various time points during the dialysis process. The concentration of paclitaxel and Tax-Chol were monitored by HPLC as described above.

In Vivo Studies in Animal Tumor Models

All studies follow established protocols approved by the Institutional Animal Care and Use Committee. Female BALB/c mice were purchased from Charles River Laboratories, monitored for 1 week upon arrival, and then placed on a folate deficient diet (Dyets Inc., Bethlehem, PA) for 2 weeks prior to treatment. For tumor implantation, 2×10^6 M109 cells in 100 μl culture media were injected subcutaneously (s.c) into BALB/c mice on the left flank, and the tumors were allowed to reach a weight of at least 32 mg at the time of initial treatment. Tumor volume was calculated based on the equation $(a \times b^2)/2$, where a equaled the length and b equaled

the width of the tumor. Mice were randomly sorted into treatment groups of 5 mice each and received a single daily intraperitoneal injection for 4 days, of saline, paclitaxel in Cremophor EL (25 mg/kg), unloaded LNs (vehicle control), or Tax-Chol in either targeted or nontargeted LNs at a dose of 37 mg/kg (equimolar to paclitaxel in the Cremophor formulation). Tumor size and animal survival were monitored daily until tumor volume met early removal criteria (greater than 1 cm^3), when the mice were sacrificed by CO_2 euthanasia. Statistical analyses were performed by one-way ANOVA (SPSS) using Tukey *post hoc* tests.

RESULTS AND DISCUSSION

Liposomes and LNs are long-circulating formulations and have been shown to increase drug uptake by solid tumors due to the enhanced permeability and retention (EPR) effect (21,22). They also have lower vehicle toxicity compared to micellar formulations such as Cremophor EL. Lipid nanoparticles (LNs) are potentially suitable delivery vehicles for anticancer drugs with poor water solubility, such as paclitaxel (2). Liposomes and emulsion formulations of paclitaxel have been evaluated previously and found to have only limited drug loading capacity due to the limited lipid solubility of paclitaxel (23–25). In this study, a lipophilic prodrug of paclitaxel, Tax-Chol, was synthesized and evaluated in LN formulations both *in vitro* and *in vivo*.

The basic composition of the LN used in this study was DSPC/triolein/Chol oleate/PEG-Chol (40:40:18:2.5, mole: mole). PEG-Chol was included to reduce *in vivo* clearance of the LNs by the reticuloendothelial system and to increase the colloidal stability of LNs. For FR targeted formulations, 20% of the PEG-Chol was replaced with folate-PEG-Chol. The LNs were prepared by rapid solvent dilution, as described in "Materials and Methods." The mean diameter of the LNs obtained was ~130 nm based on dynamic light scattering. Incorporation of Tax-Chol was greater than 90% at drug-to-lipid ratio of 1:20. This represents a significant improvement over the 70% loading efficiency observed with a LN formulation of paclitaxel with a similar composition described in a previous report from this laboratory (20). This is presumably due to the increased lipid solubility of Tax-Chol compared to paclitaxel.

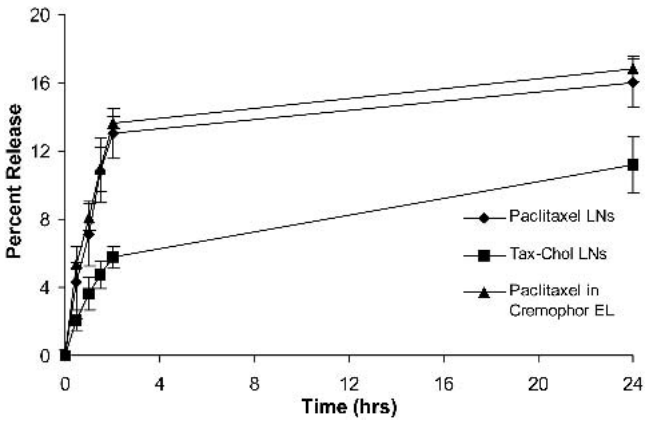

Fig. 2. Release of Tax-Chol and paclitaxel from LNs and paclitaxel in Cremophor EL, in the presence of 45 mg/ml bovine serum albumin. The formulations were dialyzed against PBS at 37°C (n = 3).

Table I. Cytotoxicity of LN Formulations Determined by MTT Assay

Cell line	IC_{50} (μM)		
	KB	M109	CHO
FR-targeted LNs	0.61	0.52	1.18
FR-targeted LNs + 1 mM free folic acid	1.21	1.12	0.82
Nontargeted LNs	2.82	2.78	1.45
Paclitaxel in Cremophor EL	0.14	0.15	0.24

LN, lipid nanoparticle; FR, folate receptor.

Stability of Tax-Chol into LNs

The physical stability of LN formulations was evaluated following storage at 4°C and 25°C for various lengths of time, using drug leakage and change in particle size as criteria. All formulations were found to be stable over a period of 6 months at both 4°C and 25°C, with very limited drug leakage (less than 10%, data not shown) and no significant increase in mean particle size distribution. In the presence of 4.5% bovine serum albumin, Tax-Chol exhibited greatly increased stability compared to paclitaxel in the same formulation and in Cremophor EL, as shown in Fig. 2. This attenuated drug release might result in a more favorable pharmacokinetic profile *in vivo* (22,24,25).

Cytotoxicity of FR-Targeted LNs

Cytotoxicity studies were carried out on FR-targeted LN formulations containing Tax-Chol (26–31). FR(+) KB and M109, and FR(−) CHO cell lines were used in MTT assays, and the results are summarized in Table I. In KB cells, FR-targeted Tax-Chol LNs exhibited greater than 4-fold reduction in IC_{50} compared to nontargeted LNs (0.61 μM *vs.* 2.82 μM). Cytotoxicity of FR-targeted LNs was reduced by 1 mM free folic acid, suggesting an important role for FR-mediated uptake. Similar results were obtained in FR(+) M109 cells. In contrast, no significant difference in IC_{50} was observed in FR(−) CHO cells between targeted and nontargeted LNs. Differences in the IC_{50} between the LN formulations and paclitaxel in Cremophor EL may be due to the limiting hy-

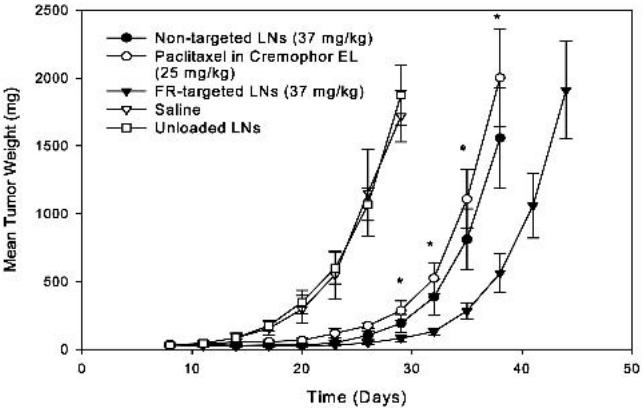

Fig. 3. Growth inhibition of subcutaneous M109 tumors in BALB/c mice by Tax-Chol in FR-targeted and nontargeted LNs and paclitaxel in Cremophor EL. Error bars represent one standard deviation (n = 5). *Denotes statistical significance between tumor growth in mice treated with targeted and nontargeted LNs ($p < 0.05$).

Table II. Tumor Inhibitory Activity of LNs and Paclitaxel in Cremophor EL in a M109 Lung Carcinoma Murine Tumor Model

Formulation	Drug dosage (mg/kg; n = 5)	Treatment schedule	Median tumor weight on day						T-C days[a]	Log cell kill[b]
			0	8	17	26	35	38		
Saline	0	q.d. × 4	0	33	170	1069	—	—	—	—
Unloaded LNs	0	q.d. × 4	0	32	155	1154	—	—	—	—
Paclitaxel in Cremophor EL	25	q.d. × 4	0	30	56	174	1108	—	8	0.7
Nontargeted LNs	37	q.d. × 4	0	37	31	013	811	1558	10	0.9
FR-targeted LNs	37	q.d. × 4	0	31	24	41	284	576	14	1.3

LN, lipid nanoparticles.
[a] T-C = Tumor growth delay value, i.e., median time for treatment group (T) and control (C) group to reach a predetermined tumor size (1000 mg).
[b] Log cell kill = (T-C)/)3.32 × tumor doubling time).

drolysis rate of Tax-Chol to paclitaxel. The duration of the study might not have allowed for sufficient time for the hydrolyzed product to elicit the same effect as paclitaxel. Nevertheless, these data do indicate that Tax-Chol is therapeutically active as a prodrug for paclitaxel and that FR-targeted Tax-Chol LNs can effectively target *in vitro* tumor cells that overexpress the FR.

In Vivo Antitumor Efficacy of the Tax-Chol LNs

An FR-targeted LN formulation of similar composition loaded with paclitaxel has been described in a recent report in this lab (20). However, *in vivo* evaluation of that formulation failed to demonstrate a therapeutic advantage for the targeted LNs, possibility due to the LNs' lack of *in vivo* stability. In the current study, FR-targeted LNs containing Tax-Chol were evaluated in subcutaneous FR(+) M109 tumors in BALB/c mice, a model frequently used for characterizing the therapeutic efficacy of paclitaxel (7–9,23). As shown in Fig. 3, mice that received FR-targeted LNs exhibited a reduced tumor growth rate compared to mice that received the nontargeted formulation or paclitaxel in Cremophor EL. Data points marked with an asterisk (*) in Fig. 3 denote a statistically significant difference between targeted and nontargeted LNs ($p < 0.05$). Statistical significance was also noted between targeted LNs and paclitaxel in Cremophor EL ($p < 0.05$), whereas nontargeted LNs were found not to be statistically different from paclitaxel in Cremophor EL ($p > 0.05$). Tumor growth delay values were calculated and are shown in Table II. Log-cell kill values of 0.7, 0.9, and 1.3 were observed for paclitaxel in Cremophor EL, nontargeted and targeted LNs at equimolar doses of 25 mg/kg (for paclitaxel) and 37 mg/kg (for Tax-Chol). Data on survival, as shown in Fig. 4, indicated that FR-targeted LNs containing Tax-Chol was more effective in prolonging the survival of tumor-bearing mice compared to nontargeted LNs, suggesting greater therapeutic efficacy due to FR targeting *in vivo*.

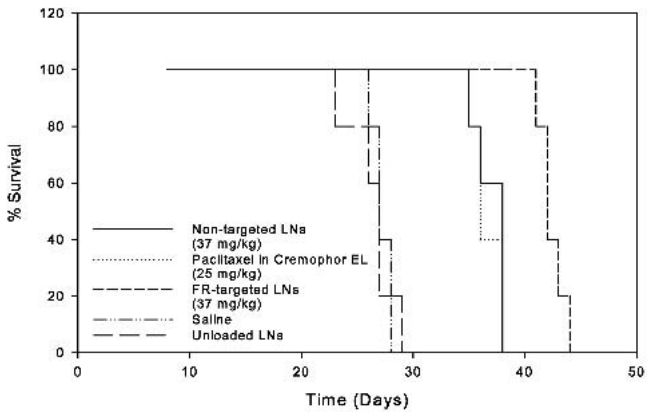

Fig. 4. Survival of BALB/c mice bearing M109 tumors in response to treatment with Tax-Chol in FR-targeted and nontargeted LN formulations and paclitaxel in Cremophor EL.

The enhanced antitumor efficacy of the FR-targeted LNs containing Tax-Chol might be due to its increased lipophilicity and stability of incorporation into the LN formulation as shown by the above *in vitro* drug release study. This provides more time for folate-conjugated LNs to reach its target site prior to the release of the drug from the formulation.

CONCLUSIONS

LNs composed of DSPC/triolein/Chol oleate/PEG-Chol (40:40:18:2) can efficiently incorporate Tax-Chol as a lipophilic prodrug of paclitaxel at a drug-to-lipid ratio of 1:20. The resulting LN formulation exhibited long-term stability and is therapeutically active against tumor cells *in vitro*. FR-targeted LNs show enhanced cytotoxicity against FR(+) but not FR(−) tumor cells and elevated antitumor activity in mice bearing FR(+) M109 tumors. In addition, FR-targeted LNs more effectively prolonged the survival of the tumor-bearing mice. The implicit *in vivo* targeting of FR(+) tumor cells might have been facilitated by the superior stability of LNs incorporating Tax-Chol compared to the parent drug. Further preclinical studies are warranted to evaluate the therapeutic potential of these LN formulations for the treatment of FR(+) tumors.

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