Synthesis and Evaluation of a Novel Lipophilic Folate Receptor Targeting Ligand

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Abstract. *Background: Folate receptor (FR)-targeted liposomes have been investigated as delivery vehicles for anticancer drugs. A novel lipophilic FR ligand, folate-glutathione-polyethyleneglycol-distearoyl phosphatidylethanolamine (F-GSH-PEG-DSPE), was synthesized, incorporated into liposomes and evaluated for FR targeting efficiency. These liposomes were then evaluated as carriers of the chemotherapy agent vincristine (VIN). Materials and Methods: F-GSH-PEG-DSPE was synthesized and FR-targeted liposomes loaded with either calcein (F-L-Calcein) or VIN (F-L-VIN) were prepared by thin film hydration followed by polycarbonate membrane extrusion and, in the case of VIN, by remote loading. To assess liposome stability, the uptake of F-L-VIN in KB (FR+) cancer cells was measured after storage under 4˚C for 3 months. Comparative pharmacokinetic studies were carried out with F-L-VIN and L-VIN (non-targeted control liposomes). Results: F-L-Calcein showed significantly higher cellular uptake in KB cells compared to non-targeted liposomes. In addition, F-L-VIN showed enhanced cytotoxicity in KB cells in vitro compared to control liposomes. Pharmacokinetic parameters indicated that both F-L-VIN and control liposomes had higher area under the curve (AUC), mean residence time (MRT), elimination half life (t1/2-β) and lower total body clearance (CL) than those of free VIN, while there were no significant differences between these liposomal formulations. Conclusion: F-GSH-PEG-DSPE is effective as a novel ligand for the synthesis of FR-targeted liposomes.*

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Folate receptor (FR) are glycoproteins with high affinity for folic acid (K_d~100 pM). Two membrane variants of the FR exist, FR-α and FR-β (1, 2). FR-α, is overexpressed in about 40% of human carcinomas. FR-β, in contrast, is expressed in its functional form on activated macrophages and myelogenous leukemia blasts (3-5). Both FRs are absent in most normal tissues.

Lee and Low (6) first reported synthesis of folate-conjugated liposomes and showed that a lengthy polyethylene glycol (PEG)-based spacer was required to bridge the folate and the lipid anchor in order to allow for FR-mediated tumor cell targeting of the liposomes. Liposomal delivery can potentially increase the efficacy of anti-cancer drugs while reducing their side effects. Subsequent studies reported at least three lipophilic derivatives, folate-PEG-distearoyl phosphatidyletha-nolamine (folate-PEG-DSPE), folate-PEG-cholesterol (F-PEG-Chol) and folate-PEG-cholesteryl hemisuccinate (F-PEG-CHEMS), which were efficacious for FR targeted delivery of liposomes (6-8). Although these folate conjugates are effective in targeting FR-expressing tumor cells, there are concerns over the tendency for the folate moiety to self-aggregate on the surface of the liposomes, resulting in reduced FR targeting efficiency. We herein report the synthesis of another lipophilic folate derivative, F-glutathione (GSH)-PEG-DSPE, which incorporates a linker that carries two negative charges. FR-targeted liposomes loaded with either calcein or vincristine (VIN) were synthesized and characterized for FR-dependent cellular uptake and cytotoxity *in vitro* and for pharmacokinetic properties *in vivo*.

Materials and Methods

Reagents. VIN was obtained from Fine Chemicals (Markham, Ontario, Canada). Egg phosphatidylcholine (EPC) and maleimide-PEG-DSPE (Mal-PEG-DSPE) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Glutathione, folic acid, N,N'-

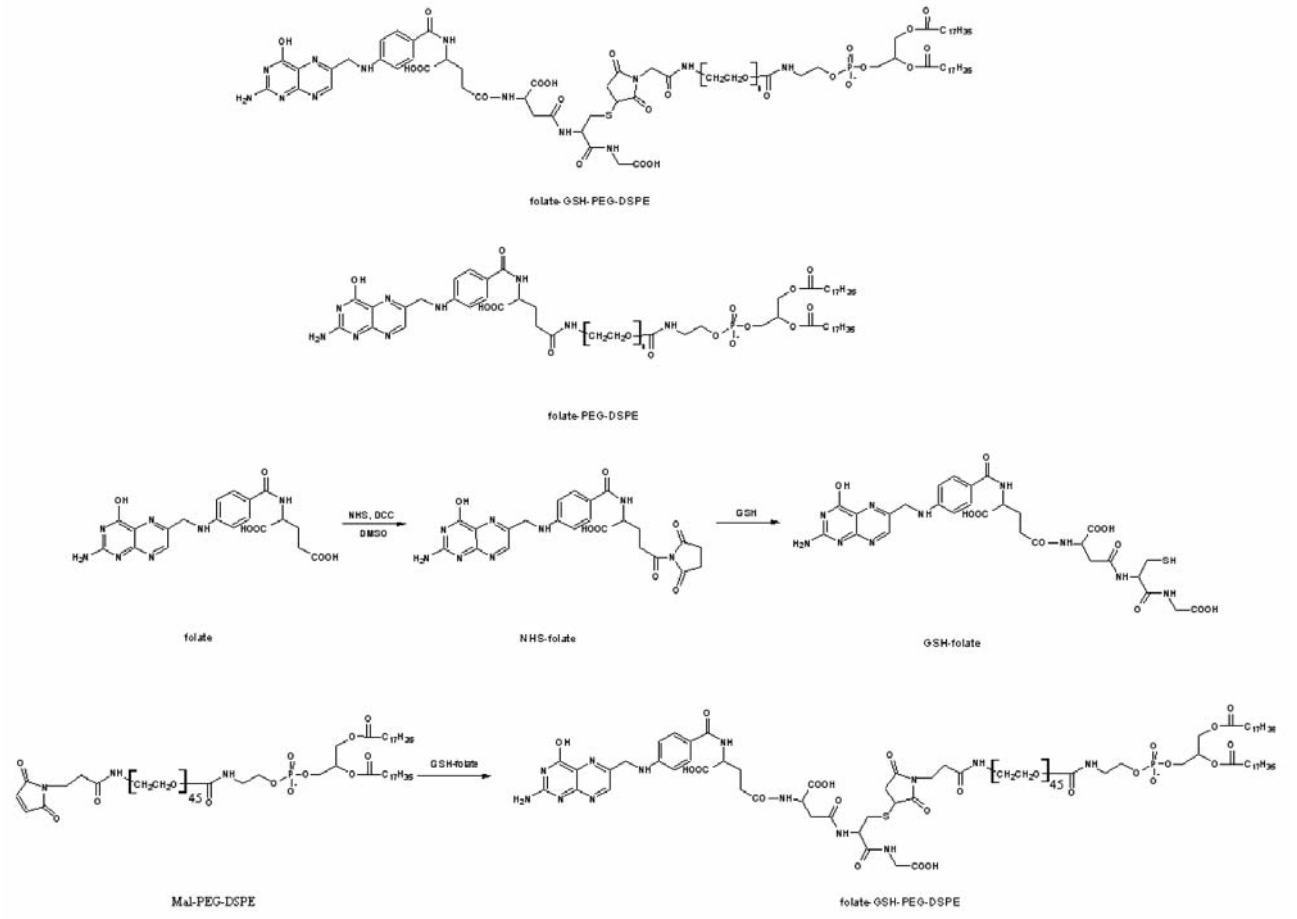

Figure 1. *Structures of F-GSH-PEG-DSPE and F-PEG-DSPE and the synthesis of F-GSH-PEG-DSPE.*

dicyclohexylcarbodiimide (DCC), N-hydroxysuccinimide (NHS), cholesterol (CHOL), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT), calcein, fluorescein-5-maleimide and Sepharose CL-4B chromatography media were purchased from Sigma-Aldrich (St. Louis, MO, USA). PD-10 desalting columns were purchased from Amersham Biosciences (Uppsala, Sweden). Polycarbonate membrane and the Lipex lipid extruder were obtained from Northern Lipids Inc. (Vancouver, BC, Canada). RPMI 1640 (folate free) tissue culture media, additives and fetal bovine serum (FBS) were purchased from Life Technologies (Rockville, MD, USA). All the reagents and solvents were of analytical or high performance liquid chromatography (HPLC) grade and were used without further purification.

Cell culture. KB cells, an FR(+) cell line derived from HeLa cervical cancer cells, were maintained in folate-free RPMI 1640 media supplemented with penicillin, streptomycin and 10% FBS under a humidified atmosphere containing 5% CO_2 at 37˚C. The folate in the FBS brings the folate concentration of the medium to that of a physiological value for human serum.

Synthesis of F-GSH-PEG-DSPE. The synthesis of the F-GSH-PEG-DSPE was carried out as shown in Figure 1. To synthesize NHS-folate, 100 mg folic acid was dissolved in 5 ml dimethylsufoxide (DMSO), along with 1.1× NHS and 1.1× DCC, as described previously (9, 10). The reaction mixture was stirred for 2 h in darkness at room temperature. The insoluble byproduct, dicyclohexylurea, was removed by filtration. Then, 1.1× GSH was added to the NHS-folate and the reaction proceeded overnight at room temperature. The product was purified by precipitation by acetonitrile, washed with diethylether and then dried to a powder. Finally, 50 nmol Mal-PEG-DSPE, dissolved in water, was added to an equamolar quality of the folate-GSH dissolved in water to produce F-GSH-PEG-DSPE.

Preparation of liposomes. The liposomes were prepared by a thin film hydration-polycarbonate membrane extrusion method. The lipid composition of the non-targeted liposomes and the FR-targeted liposomes was EPC/CHOL/mPEG-DSPE at a molar ratio of 55:40:5 and EPC/CHOL/mPEG-DSPE/F-GSH-PEG-DSPE at a molar ratio of 55:40:4.5:0.5, respectively. VIN was remote-loaded into the liposomes by a transmembrane pH gradient (11-13). Briefly, the lipids were dissolved in $CHCl_3$ and dried into a thin film by rotary evaporation followed by further drying under vacuum. The lipid film was hydrated with 2 ml of 300 mM citrate buffer (pH 4.0) for 30 min at 37˚C under rotary evaporation. The resultant suspension of multilamellar vesicles was then subjected to three cycles of freezing and thawing, and then

extruded three times through a 100 nm pore size polycarbonate membrane using a Lipex Extruder (Northern Lipids Inc.) driven by pressurized nitrogen to produce unilamellar vesicles. The external buffer was exchanged with phosphate-buffered saline (PBS, pH 7.4) by size-exclusion chromatography on a Sepharose CL-4B column. The eluted liposomes had a transmembrane pH gradient (internal: pH 4.0, external: pH 7.4). VIN·SO_4 (4 mg) was dissolved in 0.4 ml deionized H_2O and added to the liposomes at a VIN-to-lipid ratio of 1:30 (w/w), followed by a 30 min incubation at 37˚C. Residual free VIN in the liposomal preparation was removed by size exclusion chromatography on a Sepharose CL-4B column. Drug encapsulation efficiency was determined by the UV absorbance of the collected fractions. The mean diameter of the VIN liposomes was determined by dynamic light scattering using a NICOMP Model 370 Submicron Particle Sizer (Particle Sizing Systems, Santa Barbara, CA, USA). The zeta potential of the liposomes was measured by Zeta PALS (Zeta Potential Analyzer, Brookhaven Instruments Corporation, Holtsville, NY, USA). The VIN concentration in the liposomes was determined by HPLC as follows: Hypersil octadecyl silane chemically bonded to silica gel (ODS) column (5μm, 4.6 mm×200 mm); mobile phase: 0.15% diethylamine (adjusted pH to 7.2 with phosphoric acid)/methanol (volume ratio, 65/35), at a flow rate of 1.0 ml/min at room temperature; detection wavelength: 220 nm.

Calcein liposomes (F-L-Calcein and L-Calcein) were prepared similarly with the following changes. After a thin film was formed following lipid drying, 1 ml calcein solution (30 mg/ml, in PBS, pH 8.0) was added in a hydration process. Then the resultant multilamellar vesicles were subjected to three cycles of freezing and thawing and were extruded three times through a 100 nm pore size polycarbonate membrane. Finally, free calcein was removed by a Sepharose CL-4B column.

Cellular uptake of F-L-Calcein and L-Calcein. KB cells, grown as a monolayer, were suspended by a brief treatment with trypsin-EDTA (Invitrogen, Carlsbad, CA, USA) and then washed once with fresh culture medium. Aliquots of cell suspensions were incubated with L-Calcein or F-L-Calcein in folate-free RPMI medium either with or without 1 mM free folic acid as an FR blocking agent. The cells were incubated for 3 h at 37˚C. At the end of incubation, the cells were washed three times with cold PBS and then were examined under a Nikon Eclipse 800 fluorescence microscope (Melville, New York, USA) or analyzed by flow cytometry on a Beckman-Coulter EPICS XL cytometer (Beckmann-Coulter, Miami, FL, USA).

Cytotoxicity analyses. The cytotoxicity of F-L-VIN to L-VIN was determined by MTT assay as described previously (14, 15). The assay characterizes cell viability based on the mitochondrial conversion of a water-soluble tetrazolium salt (MTT) to a water-insoluble blue formazan product. The KB cells were transferred to 96-well culture plates at 2×10^4 cells per well 24 h prior to drug addition. The culture medium was then replaced with 200 μl medium with serial dilutions of F-L-VIN, with or without free folate for FR blockade, L-VIN, or free VIN. After 3 h incubation at 37˚C, the cells were washed twice with cold PBS and cultured in fresh medium for 48 h. Then, 20 μl MTT stock solution (5 mg/ml in PBS) was added to each well and the plate was incubated for 2 h at 37˚C. The medium was then removed and the blue formazan crystal converted from MTT was solubilized in 200 μl DMSO. Cell viability was then assessed by absorbance at 570 nm measured on an automated plate reader (Biorad, Hercules, CA, USA).

Table I. *Mean particle size, zeta potential and loading efficiency of F-L-VIN and L-VIN*.

Formulation	Mean particle size (nm)	Zeta potential (mV)	Loading efficiency
L-VIN	108.3±24.9	–19.88±3.85	96.8%
F-L-VIN	112.7±27.2	–15.88±4.56	99.5%

Means and standard deviations (n=3).

Pharmacokinetic studies. The plasma clearance kinetics of VIN in F-L-VIN and L-VIN were evaluated in imprinting control region (ICR) mice (Charles River Lab, Wilmington, MA, USA). The mice (three per group) received intravenous injections of different liposomal formulations at 5 mg drug/kg body weight *via* the tail vein. Blood samples were collected in heparin-treated tubes at various time-points (0.25, 0.5, 1, 2, 4, 8, 16, 24 and 48 h). The plasma was isolated by centrifugation (10 min at 5000 rpm) and then analyzed for VIN content by HPLC. During the analysis, 200 μl plasma sample was mixed with 25 μl H_3PO_4 solution (0.5 mol/L). The extraction of VIN was accomplished by the addition of 3.0 ml chloroform and vortex mixing for 1 min. The mixture was then centrifuged for 10 min at 10,000 rpm at 4˚C, after which 3.0 ml of the organic layer was transferred to a clean tube and evaporated. For HPLC sample loading, 100 μl of the mobile phase was used to reconstitute the residue and a 20 μl aliquot was injected into a C18 reverse phase column for analysis, as described above. The pharmacokinetic parameters were ontained using WinNonlin software (Pharsight, St. Louis, MO, USA) and a two-compartment model.

Results

Characterization of F-L-VIN. The F-L-VIN had a mean diameter of 112.7±27.2 nm and a relatively narrow distribution (as shown in Table I). In a typical preparation, the efficiency of remote loading of VIN into the liposomes was >95% at the drug/lipid ratio of 1:30 (w/w).

Cellular uptake of F-L-Calcein compared to L-Calcein. The uptake of calcein liposomes containing F-GSH-PEG-DSPE by the KB cells was analyzed by fluorescence microscopy (Figure 2) and by flow cytometry (Figure 3). After 3 months in storage, the mean particle size of the liposomes increased from 112.7 to 134 nm. The uptake of calcein liposomes containing F-GSH-PEG-DSPE by the KB cells was analyzed by fluorescence microscopy (Figure 2) and by flow cytometry (Figure 3). However, the liposomes containing F-GSH-PEG-DSPE retained efficiency in targeting FR-positive KB cells after 3 months storage at 4˚C, which is similar to the state in the fresh preparation. In contrast, the liposomes containing F-PEG-DSPE demonstrated diminished FR-targeting activity after 3 months storage (Figure 2).

Cytotoxicity analyses. As shown in Table II, the IC_{50} of F-L-VIN was 2.64±0.14 μM, showing significant receptor-specific cytotoxicity ($p<0.05$) when compared to L-VIN. The presence

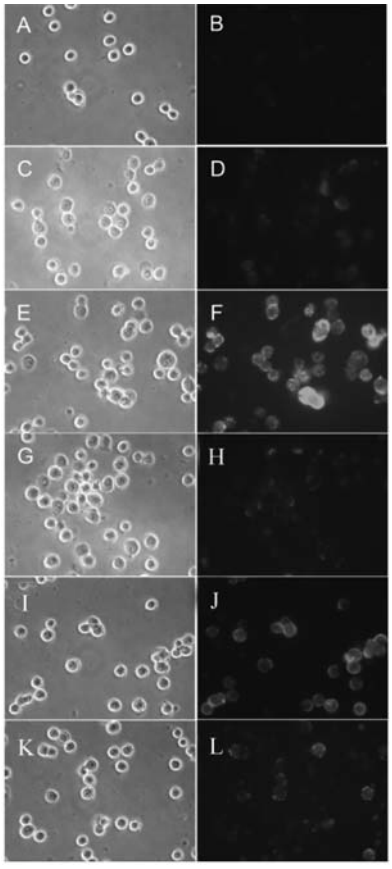

Figure 2. *Uptake of F-L-Calcein and L-Calcein by KB cells shown by fluorescence microscopy. Left panels, cells visualized in phase contrast mode; right panels, the same fields in fluorescence mode. A, B: PBS; C, D: L-Calcein; E, F: F-L-Calcein containing F-GSH-PEG-DSPE after storage at 4˚C for 3 months; G, H: F-L-Calcein containing F-GSH-PEG-DSPE plus 1 mM free folate; I, J: F-L-Calcein containing F-PEG-DSPE after storage at 4˚C for 3 months; K, L: F-L-Calcein containing F-PEG-DSPE plus 1 mM free folate.*

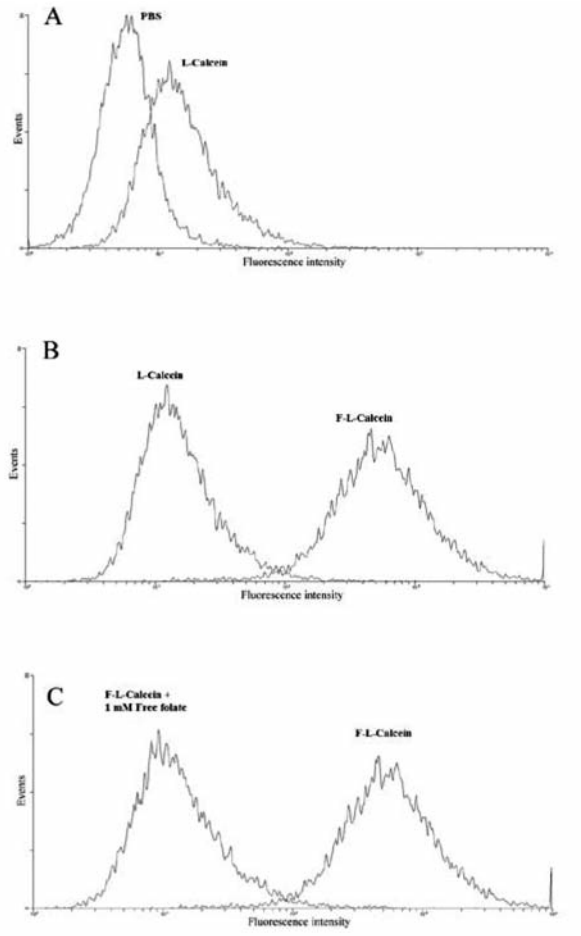

Figure 3. *Uptake of stored F-L-Calcein and L-Calcein by KB cells measured by flow cytometry.*

of 1 mM folic acid diminished the difference in IC_{50} values.

Pharmacokinetic studies. The pharmacokinetics of VIN after the administration of F-L-VIN, L-VIN or free VIN are shown in Figure 4. The free VIN was rapidly eliminated and had relatively low AUC, MRT, and $t_{1/2-\beta}$ values while CL was higher than that of liposomal VIN (Table III). The FR-targeted F-L-VIN containing F-GSH-PEG-DSPE was cleared more rapidly compared to the non-targeted L-VIN.

Discussion and Conclusion

The results of the binding analysis, cytotoxicity assay and uptake study on the new folate derivative in this study correlated well with the findings of the previously synthesized ligands described in the literature (6, 8, 16). F-GSH-PEG-DSPE exhibited excellent FR-targeting properties. The F-PEG-DSPE targeted liposomes gradually lost part of their FR targeting ability upon storage at 4˚C, while the F-GSH-PEG-DSPE liposomes showed better retention of FR-targeting efficiency. This might have been due to the GSH moiety in the F-GSH-PEG-DSPE, which is ionized and increases the distance between the folate molecules on the liposome surface, thereby preventing the folate moiety from self-association and seclusion. Overall, this may benefit the FR-targeting properties of the ligand.

In the pharmacokinetic studies, the F-L-VIN showed more rapid clearance from the circulation than the non-targeted L-VIN, which was consistent with previous reports on liposomes containing F-PEG-CHEMS or F-PEG-Chol (8, 16). This might have been due to increased uptake of the liposomes by macrophages, which express low levels of FR.

Liposomes containing the novel ligand, F-GSH-PEG-DSPE are efficiently transported into KB cells (FR+) and display good physiochemical properties, as compared with liposomes containing F-PEG-DSPE and exhibit greater cytotoxicity than

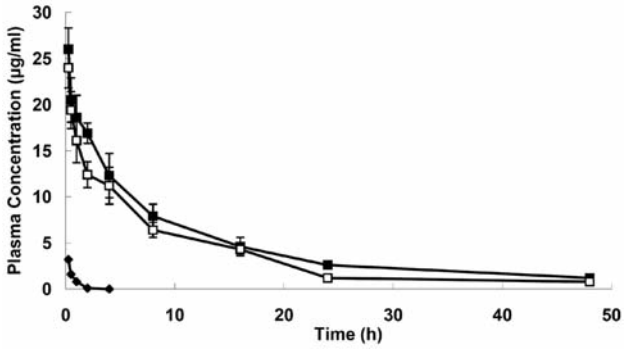

Figure 4. *Plasma concentrations of VIN in ICR mice after i.v. injection of liposomal formulations of VIN or free VIN at a dose of 5 mg/kg. Means and standard deviations (n=3). (♦) Free VIN; (□) F-L-VIN, targeted liposomes containing F-GSH-PEG-DSPE; (■) L-VIN, non-targeted liposomes.*

non-targeted L-VIN. Since F-GSH-PEG-DSPE has a greater hydrophilicity than F-PEG-DSPE, it may be a better FR-targeting ligand for the preparation of FR-targeted liposomes and nanoparticles. Further studies are warranted to validate this observation in the context of therapeutic drug delivery.

Acknowledgements

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Table II. *Cytotoxicities of F-L-VIN, L-VIN, and VIN in KB cells.*

Treatment group	IC_{50} (μM)*
F-L-VIN	2.64±0.14
F-L-VIN plus 1 mM free folic acid	8.72±0.52
L-VIN	12.7±1.02
Free VIN	0.12±0.02

Means and standard deviations (n=3). F-L-VI, targeted liposomes containing F-GSH-PEG-DSPE; L-VIN, non-targeted liposomes.

Table III. *Pharmacokinetic parameters of F-L-VIN, L-VIN, and VIN in ICR mice following i.v. administration.*

	AUC (μg•h/ml)	CL (ml/h)	$t_{1/2-\beta}$ (h)	MRT (h)
Free VIN	1.90	2.12	1.72	1.67
L-VIN	227.3	0.05	4.62	6.30
F-L-VIN	176.2	0.06	5.28	7.59

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