Novel Liposomal Gefitinib (L-GEF) Formulations

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Abstract. *Background: Gefitinib is a promising agent for the treatment of non-small cell lung cancer. The purpose of this study was to develop a novel liposomal formulation for gefitinib (L-GEF) to improve its therapeutic index. Materials and Methods: Several L-GEF formulations were prepared and characterized for their physical chemical properties and cytotoxicity. The pharmacokinetic parameters of the liposomes were determined in mice. The effect of lipid composition, transmembrane pH gradient, and incorporation of hydroxypropyl-β-cyclodextrin (HPβCD) on drug-loading efficiency, liposomal stability, and the rate of drug release were investigated. Results: The L-GEF formulation composed of hydrogenated soy phosphatidylcholine (HSPC)/cholesterol (Chol)/monomethoxy polyethylene glycol 2000-distearoyl phosphatidyl-ethanolamine (mPEG-DSPE) encapsulating 0.3 M (NH4)2SO4 and 0.1 M HPβCD (L-GEF-HSPC), had a drug-loading efficiency (DLE) of 85.5%. In vitro release studies showed that gefitinib release from L-GEF-HSPC in the presence of human plasma was slow and exhibited non-Fickian kinetics. Pharmacokinetic study in mice after i.v. bolus administration of L-GEF-HSPC showed that the area under the plasma concentration time curve (AUC) for gefitinib was 32.41 μg·h /ml and six times that of free gefitinib. The elimination half life ($t_{1/2\beta}$) of L-GEF-HSPC was 7.29 h, while that of free gefitinib was 2.26 h. Conclusion: It was shown that gefitinib can be efficiently loaded into L-GEF-HSPC composed of HSPC/Chol/mPEG-DSPE (55/40/5 mol/mol) with 0.3 M $(NH_4)_2SO_4$ and 0.1 M HPβCD as trapping agents. Compared with the free drug, L-GEF-HSPC had high drug loading, good stability, and long-circulating properties.*

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Gefitinib (Figure 1) is a selective inhibitor of the intracellular tyrosine kinase domain of the epidermal growth factor receptor (EGFR). It has shown potent antitumor activity in various xenograft models as monotherapy and when combined with other chemotherapeutics (1-5). Gefitinib was approved for clinical use against non-small cell lung cancer (NSCLC) in 2003 by the FDA, although this approval was withdrawn in 2005 due to its limited efficacy. The drug is still in clinical use in other countries (6, 7). Gefitinib is a weak base with pK_a's of 5.4 and 7.2. It is practically insoluble above pH 7, with a sharp drop in solubility between pH 4 and pH 6. It has a high affinity for human plasma proteins, which limits its achievable free drug concentration in cancer tissues (8-10). Additionally, oral gefitinib tablets (marketed as Iressa) may cause diarrhea, nausea, and vomiting. In order to improve the therapeutic index and reduce the gastrointestinal side effects, it is desirable to develop improved formulations for gefitinib.

Liposomes have gained increasing attention as drug carriers in tumor treatment because they can potentially increase the therapeutic effect while reducing the toxic effect of drugs. The bilayer structure of liposomes may be utilized for both hydrophobic and hydrophilic drugs. Many preparative methods have been developed for the loading of liposomes. Remote loading has emerged as a popular option for encapsulating therapeutic agents. Many amphipathic weakly acid or weakly basic drugs are remotely loaded successfully into preformed liposomes using various gradients (11-15), resulting in products such as Doxil™ (16). In remote loading, a gradient formed between the liposome core and the external medium drives the internalization of the drug. In this study, gefitinib liposomes (L-GEF) were prepared with high drug-loading efficiency (DLE) and good stability *via* pH gradient, with hydroxypropyl-β-cyclodextrin (HPβCD) as an additional trapping agent.

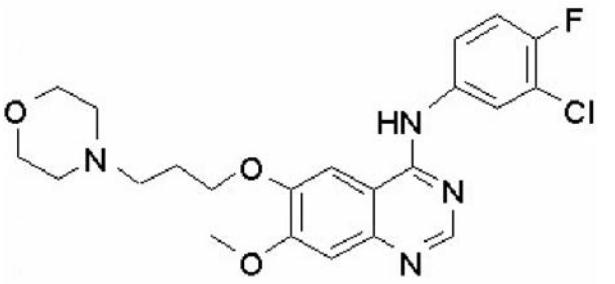

Figure 1. *Structure of gefitinib.*

Materials and Methods

Reagents. Egg phosphatidylcholine (EPC) and hydrogenated soy phosphatidylcholine (HSPC) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Cholesterol (Chol), chloroform, and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Chemicals Co. (St. Louis, MO, USA). Monomethoxy polyethylene glycol 2000-distearoyl phosphatidylethanolamine (mPEG-DSPE) was purchased from Genzyme Pharmaceuticals (Cambridge, MA, USA). Gefitinib was purchased from LC Laboratories (Woburn, MA, USA). Phosphate-buffered saline (PBS) was purchased from Fisher Scientific (Pittsburgh, PA, USA). HPβCD, Sepharose CL-4B chromatography media and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture. A549 human lung cancer cells were purchased from the American Type Culture Collection (Rockville, MD, USA). The cells were cultured in RPMI-1640 media (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA), 100 units/ml penicillin, and 100 mg/ml streptomycin. Cells were maintained at 37˚C in a humidified atmosphere containing 5% CO_2.

Preparation of L-GEF. L-GEF was prepared by two different methods, with and without pH gradient-assisted loading. A mixture of EPC or HSPC, cholesterol, and mPEG-DSPE (55/40/5 mol/mol), and gefitinib at a lipid to drug weight ratio of 20:1 were dissolved in chloroform and subsequently evaporated at 35˚C to form a thin film. The resulting lipid film was rehydrated with PBS (pH 7.4), 0.3 M $(NH4)_2SO_4$ solution, or 0.3 M $(NH_4)_2SO_4$ plus 0.1 M HPβCD, and incubated at room temperature for 30 min. The generated multilamellar vesicles (MLV) were freeze-thawed three times and extruded five times through 0.2 μm pore size polycarbonate membranes on a Lipex extruder (Northern Lipids Inc., Vancouver, BC, Canada). In order to form a transmembrane gradient, solution outside of the liposomes was removed by size exclusion chromatography (SEC) or by tangential flow diafiltration against PBS, pH 7.4, using a MicroKros Hollow Fiber Module (Spectrum Laboratories, Rancho Dominguez, CA, USA) with a molecular weight cut-off (MWCO) of 50 kDa.

Particle size determination. The size distribution of the liposomes was determined by dynamic light scattering on a NICOMP Submicron Particle Sizer Model 370 (NICOMP, Santa Barbara, CA, USA). All particle size data refer to volume-weighted averages. The zeta potentials (ζ) of the liposomes were determined on a ZetaPALS instrument (Brookhaven Instruments Corp., Holtsville, NY, USA).

Determination of DLE. The free drug was removed by SEC on a Sepharose CL-4B column. The amount of gefitinib incorporated into the liposomes was determined at 252 nm using a Shimadzu UV-160U UV-VIS Recording Spectrophotometer (17). In short, the liposomes were dissolved with 80% ethanol containing 0.1 M HCl. The DLE was calculated using the following equation (18):

$$\text{DLE (\%)} = \frac{\text{Amount of drug in L-GEFG}}{\text{Amount of gefitinib initially added}}$$

Gefitinib release experiments. The *in vitro* release of gefitinib from the liposomes was determined using cellulose membrane dialysis (MWCO 3 kDa) (19). Briefly, 0.5 ml of liposomal suspension combined with 0.5 ml of human plasma was placed in a cellulose dialysis membrane bag (Viskase Co., Chicago, IL, USA), which had been pre-hydrated by soaking in water overnight and washed with deionized water. The dialysis bag was then placed in 500 ml PBS. Drug release was monitored at 37˚C and drug release from the liposomes at 1, 2, 4, 8, 12, 24, 36, 48 and 72 h was determined at 252 nm. At the given time points, 20 μl aliquots were withdrawn from the dialysis bag for analysis. At 24 h and 48 h, the external medium was removed and replaced with fresh buffer in order to maintain sink condition.

Stability of the liposomal formulation. Formulation stability was evaluated at 4˚C for a period of 30 days. The DLE, particle size, and zeta potential of the liposomes were determined on days 0 and 30.

Cytotoxicity assay. In brief, A549 cells (0.5×10^4) were plated in 96-well cell culture plates in RPMI-1640 medium containing 10% fetal bovine serum for a total volume of 100 μl per well. Free gefitinib was dissolved in dimethyl sulfoxide (DMSO). Subsequently, 100 μl medium containing 1:4 serial dilutions of L-GEF and free gefitinib were added to the cells. The cells were incubated at 37˚C for 48 h before adding 20 μl MTS solution to each well and the cells with incubation at 37˚C for an additional hour. The absorbance was measured at 490 nm on an automated plate reader. The half-maximal inhibitory concentration (IC_{50}) of L-GEF and free gefitinib were calculated.

Pharmacokinetic studies. Imprinting control region (ICR) mice (Charles River Lab, Wilmington, MA, USA) were treated with free gefitinib or L-GEF at 10 mg/kg body weight *via* tail vein injection. The free gefitinib was dissolved in DMSO and further diluted with 4.5% glucose (pH 4) to 3.5 mg/ml for *i.v.* administration. Blood samples were collected in heparin-containing tubes at 0.25, 0.5, 1, 2, 4, 8, 12, 24, and 48 h. Plasma was isolated by centrifugation at 4000 ×*g* for 10 min and stored at –20˚C. Gefitinib in plasma was extracted and analyzed by electrospray ionization liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) with a method previously reported (20). WinNonlin Version 3.2 (Pharsight Co., CA, USA) was used to determine pharmacokinetic parameters, including area under the curve (AUC), total body clearance (CL), and plasma half-life.

Results

Characterization of L-GEF. L-GEF was prepared by thin film hydration or thin film hydration with pH gradient. In order to obtain a stable liposomal formulation of gefitinib with high DLE, different lipid compositions, including EPC/Chol/mPEG-

Table I. *Effect of lipid composition and internal salt solution on drug loading efficiency (DLE), zeta potential, and particle size.*

Liposome	Lipid composition, internal solution	Particle size (nm)	Polydispersity index	Zeta potential	DLE (%)
L-GEF-EPC-1	EPC/Chol/mPEG-DSPE, pH 7.4 PBS	84.5±8.7	0.115±0.048	–13.7±1.1	24.3±1.1
L-GEF-EPC-2	EPC/Chol/mPEG-DSPE, 0.3 M $(NH_4)_2SO_4$	88.2±10.1	0.125±0.074	–14.2±2.1	45.4±0.7
L-GEF-EPC-3	EPC/Chol/mPEG-DSPE, 0.3 M $(NH_4)_2SO_4$ plus 0.1 M HPβCD	136.2±12.4	0.133±0.065	–15.3±1.7	98.1±0.8
L-GEF-HSPC-1	HSPC/Chol/mPEG-DSPE, 0.3 M $(NH_4)_2SO_4$	149.7±15.3	0.038±0.018	–15.6±1.5	71.6±1.7
L-GEF-HSPC-2	HSPC/Chol/mPEG-DSPE, 0.3 M $(NH_4)_2SO_4$ plus 0.1 M HPβCD	155.4±14.9	0.047±0.026	–17.9±1.8	85.5±1.2

Data represent the mean±SD (n=3).

DSPE and HSPC/Chol/mPEG-DSPE, and internal salt solution gradients including PBS (pH 7.4), 0.3 M $(NH4)_2SO_4$, and 0.3 M $(NH_4)_2SO_4$ with 0.1 M HPβCD were utilized. The DLE, zeta potential, and particle size of different liposomal formulations of gefitinib are shown in Table I. The zeta potentials of all liposomes were in the range of –13 to –18 mV. L-GEF-1 prepared by thin film hydration without pH gradient had very low DLE (24.3%). It was found that the pH gradient and HPβCD added to the internal solution, significantly increased the DLE. L-GEF-EPC-3 composed of EPC/Chol/mPEG-DSPE driven by 0.3 M $(NH_4)_2SO_4$ gradient loading with 0.1 M HPβCD (L-GEF-EPC) led to the highest DLE (98.1%). The L-GEF composed of HSPC/Chol/mPEG-DSPE driven by 0.3 M $(NH_4)_2SO_4$ gradient with 0.1 M HPβCD (L-GEF-HSPC-2) had a DLE of 85.5%. The mean particle sizes of L-GEF-EPC-3 and L-GEF-HSPC-2 were 136.2 nm and 155.4 nm respectively.

In vitro release studies. The release profiles of gefitinib from L-GEF-EPC-3 and L-GEF-HSPC-2 in human plasma are shown in Figure 2. No significant burst effect was observed. The release of gefitinib from L-GEF-HSPC-2 over a time period of 24 h was <25%, while at 72 h, the liposomes retained over 60% gefitinib. The *in vitro* release kinetics of gefitinib from L-GEF-EPC-3 and L-GEF-HSPC-2 were determined. To investigate the release kinetics, data obtained from the *in vitro* release tests were fitted to zero-order and first-order models. The result suggests that the drug transport out of the liposomes was driven mainly by a diffusion-guided mechanism.

Stability study of L-GEF-EPC and L-GEF-HSPC. Liposome stability was evaluated after one month at 4˚C. As shown in Table II, the L-GEF-HSPC-2 was stable over this period, with negligible changes in DLE, particle size, and zeta potential. L-GEF-EPC-3 exhibited significant changes over one month at 4˚C; the drug retention decreased from 98.1% to 83.4%.

Cytotoxicity of L-GEF formulations. Cytotoxicity of various gefitinib formulations was determined in A549 cells by the MTS assay. The IC_{50} values for free gefitinib, L-GEF-EPC-3, and L-GEF-HSPC-2 in A549 cells are summarized in Table III.

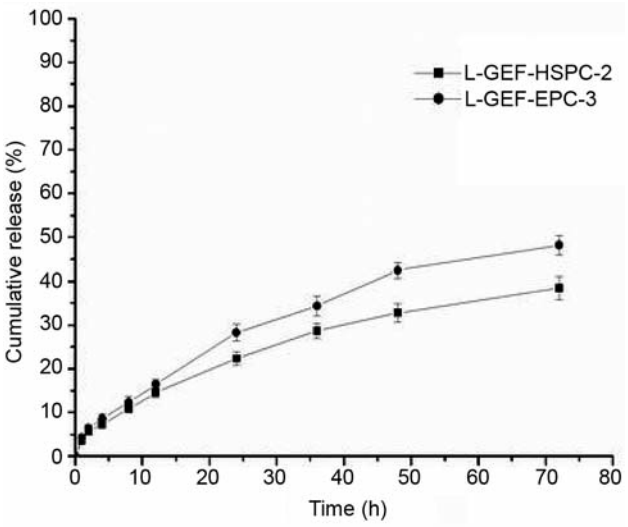

Figure 2. *In vitro release profile of gefitinib from liposomal formulations, L-GEF-EPC-3 and L-GEF-HSPC-2. Data represent the mean±SD (n=3).*

In A549 cells, free gefitinib led to the highest toxicity. In contrast, L-GEF had reduced cytotoxicity in A549 cells. L-GEF-HSPC-2 exhibited minimal cytotoxicity, indicating stable sequestration of gefitinib by liposomal encapsulation.

Pharmacokinetic study. The pharmacokinetic properties of L-GEF-HSPC-2 were studied in mice. Gefitinib plasma concentration-*versus*-time profiles are shown in Figure 3. The data showed that the pharmacokinetics of gefitinib fitted a two-compartment model. Pharmacokinetic parameters were calculated using WinNonlin and are shown in Table IV. Compared to free gefitinib, the L-GEF was cleared from plasma at a slower rate. The elimination half life ($t_{1/2\beta}$), the AUC of L-GEF-HSPC-2, the CL, and the distribution volume at steady state (V_{ss}) of L-GEF-HSPC-2 were 7.29 h, 32.41 μg·h /ml, 0.31 l/kg, and 2.83 l/h/kg respectively, while those of free gefitinib were 2.26 h, 5.21 μg·h /ml, 1.92 l/kg and 4.53 l/h/kg respectively. These parameters indicate that L-GEF-HSPC remained for an extended period of time in plasma circulation.

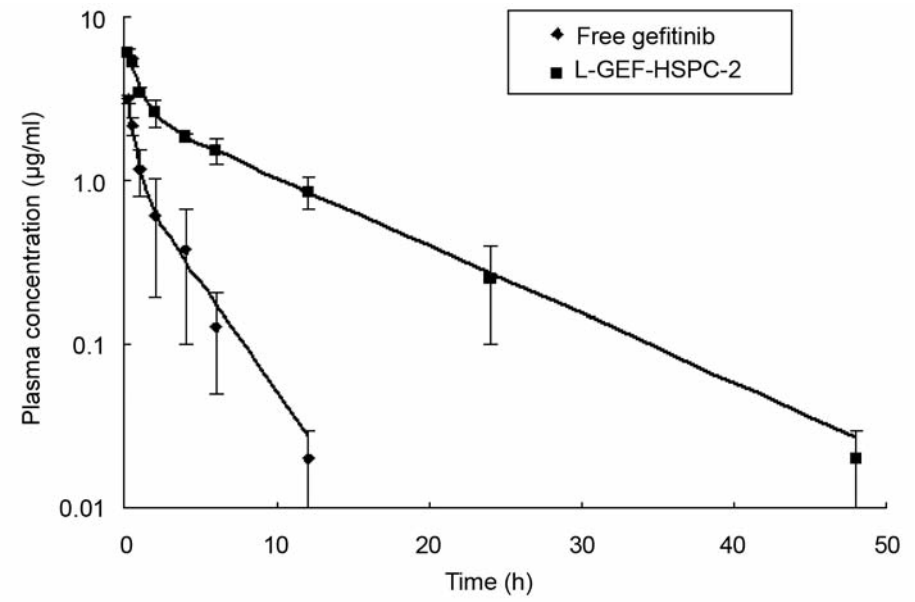

Figure 3. *Plasma clearance of gefitinib in mice. Free gefitinib or L-GEF was given by i.v. bolus injection at a dose of 10 mg/kg. Values are means±SD (n=3).*

Table II. *Stability of liposomal formulations of gefitinib at 4˚C.*

Formulation	DLE		Size (nm)		Zeta potential (mV)	
	Day 0	Day 30	Day 0	Day 30	Day 0	Day 30
L-GEF-EPC-3	98.1±0.8	83.6±2.3	136.2±15.7	159.6±17.2	–15.3±1.7	–13.5±1.4
L-GEF-HSPC-2	85.5±1.2	85.1±1.5	155.4±13.1	162.9±18.4	–17.9±1.8	–16.0±1.3

Data represent the mean±SD (n=3).

Discussion

Gefitinib, a poorly soluble drug, is used in the treatment of locally advanced or metastatic NSCLC for patients who have previously received chemotherapy. In this study, the loading efficiency of gefitinib for liposomal formulations was optimized by a pH gradient-driven loading method. The results indicated that liposomes containing gefitinib prepared by thin film hydration and passive loading have low DLE and stability. The use of a pH gradient facilitated efficient loading of the L-GEF. Gefitinib can be entrapped to the interior of liposomes by forming a sulfate salt. The transmembrane pH-gradient drove the drug into the liposomal core, which also leads to increased liposomal stability and slow drug release.

In our study, we found that addition of HPβCD to the internal medium can further increase the DLE of L-GEF. HPβCD, a β-cyclodextrin derivative, has been shown to stabilize a wide variety of compounds *via* inclusion complex formation (21). It has high water solubility and an excellent safety profile, which lead to application in systemic

Table III. *Cytotoxicity of liposomal gefitinib formulations to A549 cells. Cytotoxicity was determined using MTS assay as described in the Materials and Methods. IC_{50} is the half maximal inhibitory concentration. Data represent the mean±SD (n=4).*

IC_{50} (μM)	A549
Free GEF	10±7
L-GEF-EPC-3	149±28
L-GEF-HSPC-2	205±12

Table IV. *Pharmacokinetic parameters of free gefitinib and liposomal formulation.*

	$T_{1/2\beta}$ (h)	V_{ss} (L/kg)	AUC (h·μg/ml)	CL (L/h/kg)	MRT (h)
L-GEF-HSPC-2	7.29	2.83	32.41	0.31	9.18
Free gefitinib	2.26	4.53	5.21	1.92	2.35

administration, even at a high concentration of 40% for intravenous injection (22, 23). Cyclodextrins can form inclusion complexes with drug molecules in aqueous media, hence increasing drug solubility (24).

The pharmacokinetic studies showed that the AUC of L-GEF-HSPC-2 was six times that of free gefitinib. L-GEF-HSPC-2 had an extended circulation time in blood, which could lead to higher L-GEF-HSPC-2 accumulation in the cancer tissue *via* the enhanced permeability and retention (EPR) effect, and potentially an increased therapeutic index.

In conclusion, gefitinib can be efficiently incorporated into liposomes by a novel loading method with a pH gradient and HPβCD as a trapping agent. The resulting L-GEF has high DLE, good stability, and long circulation properties.

Acknowledgements

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