A Liposomal Delivery Vehicle for the Anticancer Agent Gossypol

GUANGXI ZHAI[1,2], JUN WU[2], XIAOBIN ZHAO[2], BO YU[2], HONG LI[2], YANHUI LU[2], WEIPING YE[3], YOUNG C. LIN[3,4] and ROBERT J. LEE[2,4]

[1]*College of Pharmacy, Shandong University, Jinan 250012, P. R. China;*
[2]*Division of Pharmaceutics, College of Pharmacy,*
[3]*Laboratory of Reproductive and Molecular Endocrinology, College of Veterinary Medicine and the*
[4]*Comprehensive Cancer Center and Center for Affordable Nanoengineering of Polymeric Biomedical Devices, The Ohio State University, Columbus, OH 43210, U.S.A.*

Abstract. *Background: Gossypol has recently been identified as a potential anticancer agent. In this study, a novel liposomal formulation is evaluated for delivery of gossypol. Materials and Methods: Gossypol was incorporated into liposomes. The liposomes were characterized for physical and chemical properties and for in vitro cytotoxicity. Results: Gossypol was stably encapsulated in these liposomes and exhibited cytotoxicity towards cancer cells. Conclusion: Liposomes can be used effectively as gossypol carriers.*

Gossypol (GP) is a naturally occurring polyphenolic compound extracted from cotton plants and was initially investigated as an antifertility agent (1-4). More recently, GP has been shown to possess antineoplastic activity against a variety of malignant cell types both *in vitro* and *in vivo* (5-9). In addition, it has been proven that GP has the ability to modulate multidrug resistance and enhance the uptake of chemotherapeutic agents into multidrug-resistant cell lines (10, 11). For the time being, GP, as an antifertility agent, is administered by the oral route. However, its oral bioavailability, at 30.9% in dogs (12), 60% in rats (13) and 14.3% in mice, is relatively low and variable (14). In a clinical trial on breast cancer, oral GP led to serious side- effects, such as nausea, fatigue, emesis, taste sensation, diarrhea and dermatological toxicity in patients (15). Furthermore, it has been confirmed in rats that *in vivo*, GP targets the epididymis, disturbing both the structure and function of this organ, and presumably disrupts sperm maturation (16). In addition, GP has only limited solubility in water, which means a delivery vehicle is needed if this drug is to be administered systemically. In order to alleviate the serious side-effects of GP associated with oral administration and achieve improved biodistribution *in vivo*, a safer and more effective formulation of GP is highly desired for further investigation of this anticancer agent.

Liposomes are spherical vesicles composed of a bilayer membrane encapsulating an aqueous core. They can potentially be used as drug carriers. Some liposomal formulations have already been approved for clinical use (17, 18) and many more are currently under preclinical or clinical investigation (19). The hydrophobic property of the liposomal bilayer allows incorporation of hydrophobic chemotherapeutic agents, such as paclitaxel (20, 21) and docetaxel (21), and the hydrophilic property of the aqueous core allows encapsulation of water-soluble compounds, such as doxorubicin and daunorubicin (17, 18). The liposomal delivery of these anticancer agents has been reported to improve solubility, prolong circulation time, alter biodistribution *in vivo*, and may reduce side-effects for these compounds.

D-alpha-tocopheryl polyethylene glycol succinate (TPGS) has been utilized in numerous drug carrier formulations in recent years (22, 23, 24). Because of its amphiphilic structure, it can be used as an emulsifier and vehicle for lipid-based drug delivery systems such as microemulsion (22), nanoparticle (23, 24), micelle (25) and solid dispersion (26-28). To date, TPGS has not been used as a component in liposomal formulations.

In this paper, we report a novel formulation using TPGS as a lipid component in liposome preparation. GP was stably incorporated to the TPGS-containing liposomes. The preparation, characterization and cytotoxicity of GP-loaded liposomes were studied.

Correspondence to: Robert J. Lee, Ph.D., College of Pharmacy, 500 W. 12th Ave., Columbus, OH 43210, U.S.A. Tel: +1 6142924172, Fax: +1 614292 7766, e-mail: lee.1339@osu.edu; Young C. Lin, DVM, Ph.D., College of Veterinary Medicine, NCI Comprehensive Cancer Center, 1900 Coffey Road, Columbus, OH 43210, U.S.A. Tel: +1 6142929706, Fax: +1 6142927599, e-mail: lin.15@osu.edu

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Table I. *Characterization of gossypol-liposomes of different compositions.*

Formulation composition (molar ratio)	Entrapment efficiency (%)	Drug concentration in liposomes (mg/ml)	Average particle diameter (nm)	Zeta potential (mV)
A. EPC:GP=10:1	83.55±2.11	1.96±0.15	104.83±7.88	–52.89±0.98
B. EPC:CH:GP=10:2:1	86.84±1.79	2.02±0.17	106.7±10.22	–58.37±1.01
C. EPC:TPGS:GP=10:0.5:1	78.73±2.23	1.85±0.21	97.94±9.44	–43.10±1.34
D. EPC:CH:TPGS:GP=10:2:0.5:1	90.67±3.44	2.16±0.14	101.9±8.34	–47.04±1.03
E. EPC:PEG-DSPE:GP=10:0.5:1	90.16±2.87	2.11±0.16	94.33±6.53	–26.01±0.71
F. EPC:CH:PEG-DSPE:GP=10:2:0.5:1	96.68±3.11	2.25±0.11	96.76±11.43	–29.49±0.89

EPC: egg phosphatidylcholine; GP: gossypol; CH: cholesterol; TPGS: D-α-tocopheryl polyethylene glycol succinate; PEG-DSPE: polyethylene glycol distearoyl phosphatidylethanolamine.

Materials and Methods

Reagents. GP acetic acid was purchased from Sigma Chemical Co. (St. Louis, MO, USA), TPGS was purchased from Eastman Chemical (Kingsport, TN, USA), egg phosphatidylcholine (EPC), cholesterol (CH) and methoxy-polyethylene glycol (M.W. 2000) distearoylphosphatidylethanolamine (mPEG-DSPE) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were analytical or HPLC grade.

Cell culture. The KB human oral carcinoma cell line, which has been identified as a contaminant of the HeLa cell line, and the MCF-7 breast carcinoma cell line were cultured as a monolayer in RPMI-1640 media (Life Technologies, Inc., Bethesda, MD, USA), supplemented with 50 μg/ml penicillin, 50 μg/ml streptomycin and 10% fetal bovine serum (FBS), and maintained in a humidified atmosphere containing 5% CO_2 at 37˚C.

Preparation of GP-loaded liposomes. Liposomes were prepared by polycarbonate membrane extrusion method, as described previously (29). Briefly, lipid ingredients including egg phospholipids, PEG-DSPE, TPGS, cholesterol and GP at different ratios were dissolved in CH_2Cl_2 and dried on a rotary evaporation in a round-bottom flask. The lipid film was further dried under vacuum and hydrated with pH 7.4 phosphate-buffered saline (PBS). The lipid suspension was then extruded 3 times each through 0.2 μm and then 0.1 μm pore size polycarbonate membranes on a Lipex lipid extruder from Northern Lipids Inc. (Burnady, BC, Canada) driven by high pressure nitrogen.

Entrapment efficiency. Liposomes were purified by size-exclusion chromatography on a Sepharose CL-4B column (Sigma-Aldrich Inc.) to remove free GP. After disruption of the liposomes with ethanol, the amount of entrapped GP was determined by absorption at 371 nm on a Shimadzu UV-Vis spectrophotometer. The entrapment efficiency was calculated as the ratio of amount of the drug incorporated in liposomes to the total drug added during liposome preparation.

Size distribution. Liposomal size distribution was determined by dynamic light scattering on a NICOMP Submicron Particle Sizer Model 370 (Nicomp, Santa Barbara, CA, USA). Liposome suspension (100 μl) was diluted to 1 ml with distilled water for the measurement. All measurements were performed at 25˚C.

Zeta potential. Zeta Potential Analyzer (Brookhaven Instruments Corporation, Holtsville, NY, USA) was used to determine zeta potentials of liposomal samples. Liposome suspension (100 μl) was diluted to 1.5 ml with distilled water and the zeta potential was determined at 25˚C .

Stability of GP-loaded liposomes. The shelf stability of the liposomal GP was evaluated by the changes in particle size and drug entrapment efficiency over a 5, 10, 15, 20, 25 and 30 day period during storage at 4˚C. The stability of the GP-loaded liposomes in plasma was determined by the retention of drug content in the liposomes. Briefly, liposome suspensions (1 ml) were mixed with FBS (2.5ml) and dialyzed using a semi-permeable membrane (MWCO 14 kDa; Spectrum Lab, Rancho Dominguez, CA, USA). After storage at 37˚C for 1, 3, 5, 8, 16 24 and 48 h, liposomal samples were collected and dissolved in ethanol. The resulting suspension was centrifuged at 10,000 rpm for 15 min and supernatant was used to determine the drug concentration by absorption at 371 nm.

Cytotoxicity of GP-loaded liposomes. Cytotoxicity of GP-loaded liposomes was determined by the MTT assay, as described elsewhere (29). Basically, MCF-7 cells were transferred to 96-well tissue culture plates at 5×10^3 cells per well 24 h prior to drug treatment. The culture medium was then replaced with 200 μl of medium containing serial dilutions of GP formulations in non-PEGylated liposomes, TPGS liposomes, PEG-DSPE liposomes or dimethylsulfoxide (DMSO). Following 24 or 48 h incubation at 37˚C, the cells were washed twice with PBS and cultured in fresh medium for an addition 48 and 24 h, respectively. A total of 20 μl MTT stock solution (5 mg/mL) was added to each well and the plates were incubated for 4 h at 37˚C. Medium was then removed and DMSO was added to dissolve the blue formazan crystals converted from MTT. Cell viability was assessed by absorbance at 570 nm measured on a Biorad microplate reader.

Results

Preparation and characterization of GP liposomes. GP liposomes, either non-coated, TPGS-containing or PEG-DSPE-coated, were prepared by extrusion. The compositions, entrapment efficiency, size distribution and zeta potential of different formulations are summarized in Table I. The preparation method (hydration of the drug-lipid film, followed by 5 cycles of extrusion through 0.2-μm and

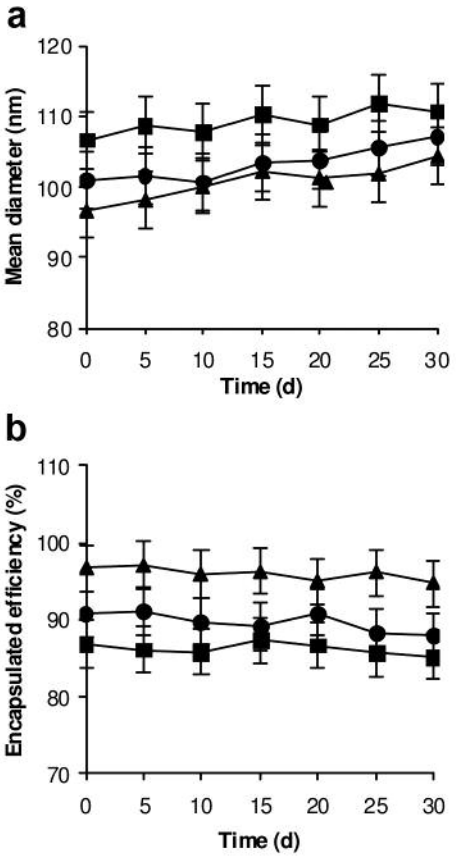

Figure 1. *Stability of gossypol-loaded liposome. a, Liposome formulation B (squares), D (circles) and F (triangles) stored at 4˚C for 5, 10, 15, 20, 25, 30 days (d) were diluted at the ratio of 1:10. The particle sizes were determined at 25˚C by dynamic light scattering on a NICOMP Submicron Particle Sizer (n=5). b, Liposome B (squares), D (circles) and F (triangles) formulations stored at 4˚C for different times were purified by size exclusion to remove free gossypol. After disruption of the liposomes with ethanol, the amount of entrapped gossypol was determined by absorption at 371 nm. The entrapment efficiency was calculated as the ratio of drug amount incorporated in liposomes to the drug amount added in preparation of liposomes. Data represent the mean±SD.*

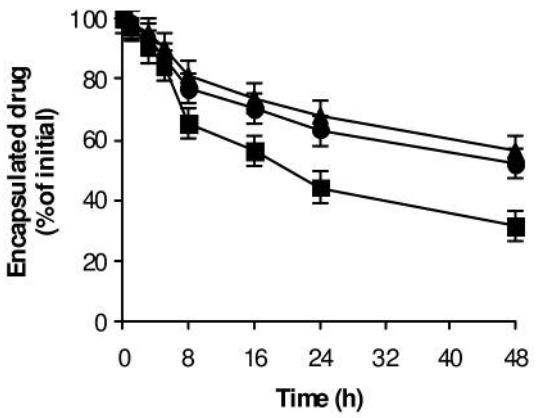

Figure 2. *Stability of gossypol-loaded liposome suspensions stored with FBS at 37˚C. Gossypol retained in liposome formulation B (squares), D (circles) and F (triangles) mixed with FBS in 1:2.5 were determined after dialysis for 8, 16, 24, 32, 40 and 48 h at 37˚C (n=5). Data represent the mean±SD.*

0.1-μm polycarbonate filters) was found to be effective in generating small homogeneous vesicles, with a mean diameter of 90 to 110 nm.

As shown in Table I, among all the formulation studied, liposomes without cholesterol, namely EPC/ TPGS (78.73%) and EPC/PEG-DSPE (90.16%) formulations, had a slightly lower encapsulation efficiency compared with other formulations. The addition of cholesterol improved the ability of liposomes with different composition to encapsulate GP, making more drug molecules into liposome vesicles; encapsulation efficiencies of the above formulations increased to 90.67% and 96.68%, respectively.

Interestingly, zeta potentials of different liposomal formulations were related to their compositions, as shown in Table I. Addition of TPGS and PEG-DSPE into phospholipid (EPC) film produced different effects on the zeta potential of liposomes. Addition of TPGS or PEG-DSPE into the lipid bilayer significantly reduced the values of zeta potential of liposomes, as demonstrated by liposome formulation C (–43.10 mV) and liposome formulation E (–26.01 mV), compared to –52.89 mV for the liposomes containing EPC and GP without TPGS or PEG-DSPE. These results indicated similar effects of TPGS and PEG-DSPE on zeta potential of liposomes.

Stability of liposomes. In order to compare the effect of composition on stability of GP-loaded liposomes, formulations B, D and F were investigated after storage at different conditions. As shown in Figure 1, there were no significant changes in the mean diameter or drug retention of liposomal formulations B, D and F in the hydrated state stored at 4˚C for 30 days. The release of drug from liposomes (formulations B, D and F) in FBS at 37˚C were 35%, 25% and 20% of the initial drug amount after 8 h of incubation, respectively, as shown in Figure 2. After 24 h of incubation with FBS at 37˚C, 45%, 63% and 68% of the initial drug amount were retained in liposomes for formulations B, D and F, respectively. Interestingly, although drug contents in liposome formulations B, D and F decreased after incubation with FBS at 37˚C, there were no significant changes in mean particle diameters. The results from the stability test of liposomes showed that liposome formulation D containing TPGS possessed similar properties to formulation F containing PEG-DSPE. As shown in Figure 2, about 25% of the encapsulated drug was released into FBS within 24 h for both liposomal formulations. However, for the formulation without TPGS or PEG-DSPE, almost 60% of the encapsulated drug was released under the same conditions. Therefore, both TPGS and PEG-DSPE increased the stability of liposomes in FBS at 37˚C.

Table II. *Cytotoxic activity (IC_{50}, values expressed in μM) of different preparations containing gossypol.*

Cell line	Exposure time (h)	Gossypol alone	Liposome formulation		
			B	D	F
KB	24	5.2±2.2	13.7±3.3	20.2±4.4	24.8±5.1
	48	3.1±1.5	5.4±2.0	7.1±2.6	7.7±2.4
MCF-7	24	11.4±3.1	29.9±4.6	42.6±7.2	48.3±7.9
	48	6.4±2.1	10.2±2.8	14.5±3.3	16.1±3.8

Cytotoxicity of liposomes. The cytotoxic activity of free drug or liposomes was related to the duration of exposure in cell lines. The IC_{50} of free drug and liposomes decreased significantly with the extent of incubation time with both KB and MCF-7 cell lines, as shown in Table II. IC_{50} values for the different liposome formulations were higher than that for the free drug, which indicated that the cytotoxicity was reduced due to the encapsulation. After 24 h incubation, the value of IC_{50} of liposome formulation D (TPGS) was more than that of formulation B (uncoated) in both KB and MCF-7 cell lines. However, no significant difference in IC_{50} was observed between D (TPGS) and F (PEG-DSPE) formulations after 48 h incubation.

Discussion

In order to avoid the side-effects caused by oral administration of GP and the limitation of its poor solubility in water, we studied the formulation of GP in a better tolerated and a less toxic vehicle. Among the different types of formulation examined, liposomes composed of EPC/PEG-DSPE/CH/GP (10:0.5:2:1) and of EPC/TPGS/CH/GP (10:0.5:2:1) showed the best encapsulation efficiencies. The inclusion of a small amount of cholesterol increased the amount of GP that could be loaded. The reasons may be that it reduced the fluidity of and increased the stability of lipid bilayer membranes formed by EPC (30, 31). Additionally, the inclusion of cholesterol had a favorable effect in reducing PEG chain interaction and entanglement and inhibiting a possible phase separation due to the presence of PEG-DSPE in the lipid bilayers (32).

For comparative purposes, we also prepared PEG-DSPE-containing liposomes containing GP under the same experimental conditions. Our test results confirmed the conclusions made by Yoshioka *et al*. (33) and Bardonnet *et al*. (34) that the inclusion of a certain amount of PEG-DSPE in lipid bilayers can increase loading of the lipophilic drug due to the decrease in the zeta potential of liposomes. We also showed lower cytotoxicity by PEGylated liposomes compared to conventional liposomes related to the steric effect of PEG chains, which may delay release of the drug from the liposomes.

TPGS as a solubilizer, an emulsifier, or an absorption enhancer, has been used in the preparation of microemulsion, nanoparticle, solid dispersion and micelle formulations. For example, Tocosol™ Paclitaxel is an emulsion containing TPGS as a surfactant currently in clinical development (35). In our study, TPGS was applied as one component of the liposomal formulation to compare with the frequently used component PEG-DSPE. Based on its molecular structure, composed of hydrophilic polyethylene glycol (1000) and hydrophobic D-alpha-tocopheryl, we expected it to exhibit similar properties to PEG-DSPE. This has been confirmed by data obtained in this study. On *in vitro* cytotoxicity, due to the controlled release of drug from the liposomes, TPGS- and PEG-DSPE-containing formulations exhibited higher IC_{50} values than uncoated liposome and free drug formulations after 24 h incubation. But *in vivo* treatment efficacy needs to be further studied, since the areas under the curve for liposomal and free drug are very different, as are the mechanisms of drug accumulation at the tumor site.

The differences between liposomal formulations D and F were related to the different length or molecular weight of polyethylene glycol chain exposed on the liposome surface (36); the molecular weight of the hydrophilic part was 1,000 for TPGS, but 2,000 for PEG-DSPE. As for the effect of TPGS and PEG-DSPE on the encapsulation efficiency of hydrophobic GP, they were significantly different, which may be related to their solubilizing ability.

In conclusion, liposomes possessing high loading of GP and good stability can be prepared by the method of film dispersion and extrusion, which provides a less toxic and better tolerated formulation for further study of GP as an anticancer agent. Liposomal GP containing TPGS or PEG-DSPE at the same molar ratio showed similar behaviors in decreasing zeta potential, controlling drug release from liposomes stored with serum at 37˚C and reducing cytotoxicity of liposomes. TPGS may have the potential to be used as a component for the preparation of long-circulating liposomal GP *in vivo*.

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