

Preparation and characterization of norcantharidin liposomes modified with stearyl glycyrrhetinate

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Abstract. In the current study, norcantharidin (NCTD)-loaded liposomes (LIPs) modified with stearyl glycyrrhetinate (SG; SG-NCTD-LIP) were prepared by the ethanol injection method. To increase the drug encapsulation efficiency (EE), the formulation of NCTD-LIP was optimized by single factor test and orthogonal design. The release of NCTD *in vitro* from SG-NCTD-LIP was evaluated by equilibrium dialysis. The cytotoxicity of SG-NCTD-LIP in HepG2 was investigated by MTT assay. The results revealed that the EE of liposomes was ~27.80%, the average SG-NCTD-LIP was 87.5 nm, the *in vitro* NCTD release from SG-NCTD-LIP was delayed compared with NCTD in solution and the drug-release kinetic followed a first-order model. MTT assays revealed increased cytotoxicity activity against HepG2 cells for SG-NCTD-LIP compared with free NCTD. In conclusion, SG-NCTD-LIP prepared in the present study may be a promising liposomal drug delivery system for anticancer drugs in liver-targeting therapy.

Introduction

Norcantharidin (NCTD; Fig. 1), the demethylated derivative of cantharidin obtained from the dried body of the Chinese blister beetle (*Mylabris spp.*) (1), has been used as an anticancer drug in China (2,3). NCTD acts by inducing cell death through mechanisms involving the response to DNA damage and apoptosis by activation of the protein kinase C signaling pathway (4). Like sulfonamides for carbonic anhydrase or hydroxamic acids for metalloproteinases, NCTD describes the archetypal small molecule protein phosphatase inhibitor (5) and has been used to inhibit proliferation and metastasis of

multiple types of carcinoma (6,7). Previous studies indicated that NCTD has therapeutic value in the treatment of various types of cancer, including liver cancer, when administered orally or intravenously (6,8-11). However, the application of NCTD is limited by numerous factors, including short half-life, high systemic toxicity, high incidence of adverse effects and poor bioavailability in the physiological environment (12,13). To improve the safety and efficacy of NCTD, NCTD nanoscale drug delivery systems (DDS) have been studied (14-16).

Liposomes are spheres with a lipid bilayer shell prepared with a variety of phospholipids, which have been extensively studied since the 1960s (17-19). Cholesterol is an additional compound in the liposomal structure that could regulate the fluidity of phospholipid membrane and may control the retention of drugs (20). Therefore, liposomes may be used as nanoscale vehicles for the administration of drugs. With good biocompatibility and low toxicity, liposomes have been revealed to enhance the therapeutic activity of numerous anticancer drugs (21-23). However, conventional liposomes have certain disadvantages, including the uptake by the reticuloendothelial system (RES) (24,25), the lack of tumor-specificity and insufficient uptake at tumor sites. Ligand-targeted liposomes, where specific ligands are used to modify liposomes, have demonstrated the potential to increase therapeutic efficacy and reduce adverse effects through the interaction between a specific ligand and the target molecule, and facilitating the receptor mediated endocytosis of liposomes (26).

Glycyrrhetic acid (GA) may be obtained by hydrolysis of glycyrrhizic acid, extracted from the roots of liquorice (*Glycyrrhiza glabra*) (27). GA possesses many pharmacological and biological relevant activities, including anti-inflammatory, antiallergic, antiulcer, antioxidant, antihepatotoxic, antineoplastic and antiviral activities (28-30). Studies have reported that specific GA binding sites may be located on the cell membrane of hepatocytes (31,32). GA and its derivatives may be used as ligands targeting the liver (33,34). Stearyl glycyrrhetinate (SG; Fig. 2), the stearyl ester of 18- β -glycyrrhetic acid, has been demonstrated to improve antiviral effects, reduce inflammation and suppress allergies in the pharmaceutical and cosmetic industry (35,36). Compared with GA, SG has an increased compatibility as its hydrophobic tail may be adsorbed into the lipid layer of liposomes while exposing the hydrophilic GA moiety at the surface.

The objective of the current study was to prepare NCTD-loaded liposomes modified with SG (SG-NCTD-LIP)

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by ethanol injection. Single factor test and orthogonal design were used to optimize the formulation of SG-NCTD-LIP. The characterization of the prepared liposomes included physical morphology, particle size and encapsulation efficiency (EE). Equilibrium dialysis was used to investigate *in vitro* release characteristics of SG-NCTD-LIP. Furthermore, *in vitro* cytotoxicity of SG-NCTD-LIP in HepG2 cells was determined by MTT assay.

Materials and methods

Materials. NCTD (>99%) was purchased from Sunray Pharmaceutical Co., Ltd. (Suzhou, China). SG (>98%) was obtained from Xi'an Realin Biotechnology Co., Ltd. (Xi'an, China). Egg phosphatidylcholine (EPC) was supplied by Lipoid GmbH (Ludwigshafen, Germany). Cholesterol was purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). HPLC-grade acetonitrile was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). All other chemicals used in this study were of analytical grade.

Preparation of SG-NCTD-LIP. Liposomes were prepared by ethanol injection method (Fig. 3) (37,38). EPC (0.1-0.4%), cholesterol (0.03-0.20%) and SG (0.04%) were dissolved in 2 ml absolute ethanol. The aqueous phase was prepared by dissolving NCTD (0.02-0.08%) in 15 ml PBS (pH 7.0) and heating in a water bath at 37°C. The 2 ml ethanol phase was immediately injected into the heated aqueous solution through a fine needle under magnetic stirring. The liposome dispersions were incubated at between 50°C for 30 min with gentle stirring.

Determination of NCTD content and EE. SG-NCTD-LIP was separated from the free drug by equilibrium dialysis (the dialysis membrane bag molecular weight cut-off of 8,000-14,000). The liposome fraction was obtained and the liposomes were ruptured in methanol for drug solubilisation. The resulting solution was sonicated (150 W at 25°C) for 5 min and filtered through polytetrafluorethylene membranes (0.22 μm). The drug concentration was determined using a reversed-phase high-performance liquid chromatography (RP-HPLC) system (LC-20AT; Shimadzu Corporation, Kyoto, Japan) at 25°C and 98% NCTD was used as a quantification standard. The mobile phase was acetonitrile/water (10:90, v/v; pH 3.1) and an isocratic elution was performed using a WondaCract ODS-2 column (5 μm , 4.6x250 mm; Shimadzu Corporation) with a flow rate of 0.8 ml/min. NCTD was detected at 220 nm. The EE was determined by dividing the amount of drug in the liposome fraction by the amount of drug in the total fractions.

Optimization of SG-NCTD-LIP formulation. Using the ethanol injection method, several factors were trialed to achieve optimal formulation, including NCTD-phospholipid mass ratio (factor A), phospholipid concentration (factor B), incubation temperature (factor C) and cholesterol-phospholipid mass ratio (factor D) during the fabrication process. Only one factor was replaced in each series of experiments. When changing the amount of phospholipids, the NCTD-phospholipid mass ratio was 1:5, cholesterol-phospholipid mass ratio was 1:7

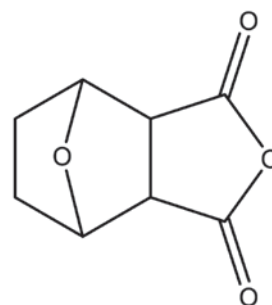


Figure 1. Chemical structure of norcantharidin.

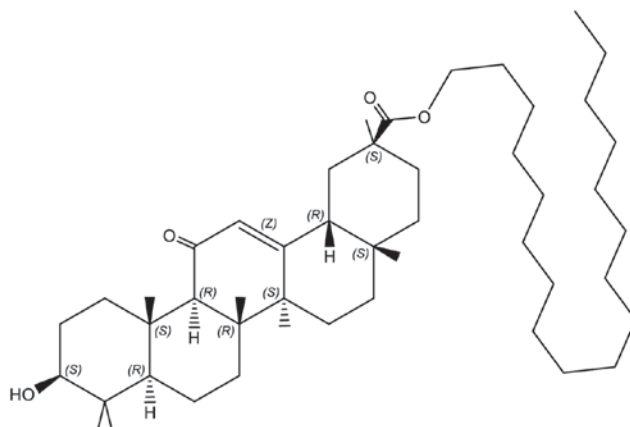


Figure 2. Chemical structure of stearyl glycyrrhinate.

and incubation temperature was 50°C. When changing the amount of NCTD, the phospholipid concentration was 0.24%, cholesterol-phospholipid mass ratio was 1:7 and incubation temperature was 50°C. When changing cholesterol-phospholipid mass ratio, the phospholipid concentration was 0.24%, NCTD-phospholipid mass ratio was 1:20 and incubation temperature was 50°C. When changing incubation temperature, the phospholipid concentration was 0.24%, NCTD-phospholipid mass ratio was 1:20 and cholesterol-phospholipid mass ratio was 1:5. Based on the investigation of factors, the four aforementioned factors were selected, and three levels of each factor were designated for the orthogonal design, with the EE as the investigating indicator to screen the formulation.

SG-NCTD-LIP size and polydispersity index (PDI). The particle size and PDI of SG-NCTD-LIP were determined at 25°C by dynamic light scattering (Nano-ZS; Malvern Instruments, Ltd., Malvern, UK). Prior to measurement, the liposome dispersions were diluted 10 times with distilled water. Measurements were performed in triplicate on independent formulations at a detection angle of 90°.

Transmission electron microscopy (TEM) measurement of SG-NCTD-LIP. A diluted SG-NCTD-LIP sample (0.018 mg/ml; 10 μl) was placed on a copper grid and air-dried at room temperature. Subsequently, a drop of 1% (w/v) aqueous solution of phosphotungstic acid was added for negative staining; the sample was dried at room temperature for 20 min. The analyses of the vesicle shape were carried out on

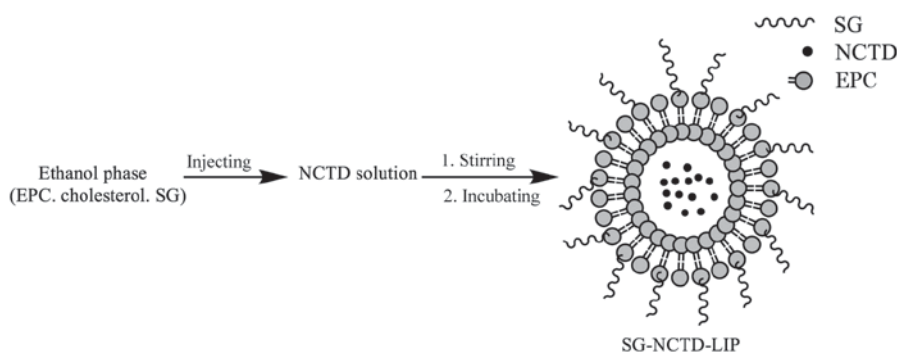


Figure 3. Ethanol injection method for preparation of SG-NCTD-LIP. The ethanol phase, containing EPC, cholesterol and SG in ethanol, was injected into the aqueous phase, containing NCTD dissolved in PBS, and incubated stirring for 30 min to yield SG-NCTD-LIP. EPC, egg phosphatidylcholine; SG, stearyl glycerol ether; NCTD, norcantharidin; SG-NCTD-LIP, NCTD-loaded liposomes modified with SG.

a JEM-2100F transmission electron microscope (JEOL, Ltd., Tokyo, Japan) with an acceleration of 150 kV.

SG-NCTD-LIP stability studies. SG-NCTD-LIP suspension stability was assessed over 3 months of storage at 4°C by means of particle size, PDI and EE measurements.

In vitro drug release study. *In vitro* drug release was performed as described previously (39). Dialysis tubing (molecular weight cut off, 8,000-14,000 g/mol) was incubated in distilled water for 12 h at room temperature. SG-NCTD-LIP (0.18 mg/ml, 8 ml) and NCTD solution (0.18 mg/ml, 8 ml) were placed in separate dialysis tubing and each incubated with stirring in 80 ml PBS (pH 7.4, release medium) at 37°C. Following 0.25, 0.5, 1, 1.5, 2, 4, 8 and 12 h incubation, 0.5 ml release medium was collected and replaced with an equal volume of fresh medium. NCTD concentration was analyzed by RP-HPLC.

Cell culture. HepG2, an immortalized cell line consisting of hepatoblastoma cells, was obtained from the Laboratory of Hepatobiliary and Pancreatic Surgery (Affiliated Hospital of Guilin Medical University, Guilin, China). Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS; both Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 U/ml penicillin and 100 µg/ml streptomycin under 5% CO₂ atmosphere at 37°C for 24 h.

Cytotoxicity study. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assays were used to study the proliferative effect of SG-NCTD-LIP. HepG2 cells were plated in 96-well culture plates at 4,000 cells/well. After overnight incubation at 37°C, cells were treated with varying concentrations (2.5, 5, 10, 20 and 40 µg/ml) of SG-modified blank liposomes (SG-LIP), NCTD solution, liposomes loaded with NCTD (NCTD-LIP) or SG-NCTD-LIP and were incubated for 48 h at 37°C. MTT (Sigma-Aldrich; Merck KGaA) solution (20 µl; 5 mg/ml) was added to each well. Following 4 h incubation at 37°C, the medium was replaced with 150 µl dimethyl sulfoxide to dissolve formazan crystals. Optical densities (ODs) were measured at 490 nm with an ELISA reader. The half-maximal inhibitory concentration (IC₅₀) was calculated using SPSS (version 22.0; IBM Corp., Armonk, NY, USA). The

inhibition ratio (IR) was calculated as follows: IR=1-OD of cells treated with sample/OD of culture medium.

Statistical analysis. Data were analyzed using SPSS software (version 22.0; IBM Corp., Armonk, NY, USA) and results are expressed as mean ± standard deviation. One-way analysis of variance followed by a Fisher's Least Significant Difference post-hoc test was used to assess the significance of the differences among various groups. Correlation coefficient (R) is a variable that demonstrated the degree of linear correlation between variables. P<0.05 was considered to indicate a statistically significant difference.

Results and Discussion

Effect of different preparation variables on the EE of SG-NCTD-LIP. The results presented in Fig. 4A identified the highest EE at 0.24% phospholipid concentration. However, excessively high phospholipid resulted in the aggregation of phospholipids and decreased EE. It was revealed that the EE of SG-NCTD-LIP was lowered from 25 to 16% when increasing the amount of NCTD; the highest EE was observed for a 1:20 NCTD-phospholipid ratio (Fig. 4B). Cholesterol is a lipid used to improve liposome stability *in vitro* and *in vivo* (40). The results indicated that the highest EE was achieved at a 1:5 cholesterol-phospholipid mass ratio (Fig. 4C). At higher ratios, cholesterol may induce rapid vesicle aggregation. Its rigidity restricts the lipid bilayer flexibility and prevents NCTD accumulation inside the vesicle. The optimum incubation temperature was determined to be 40°C (Fig. 4D).

Optimization of the SG-NCTD-LIP formulation by orthogonal design. Four factors, NCTD-phospholipid mass ratio (factor A), phospholipid concentration (factor B), incubation temperature (factor C) and cholesterol-phospholipid mass ratio (factor D) were selected as main influencing factors. Each factor was studied at three levels and the EE was selected as the investigated indicator of the formulation. The orthogonal factors and levels are presented in Table I. Nine formulations were tested, according to the L₉ (3⁴) orthogonal table. Table II displays the orthogonal test results and the variance analysis is presented in Table III. According to the extremum values (R) in Table II, the importance of the factors may be ranked as A>B>C>D. The optimum formulation composition was identified as

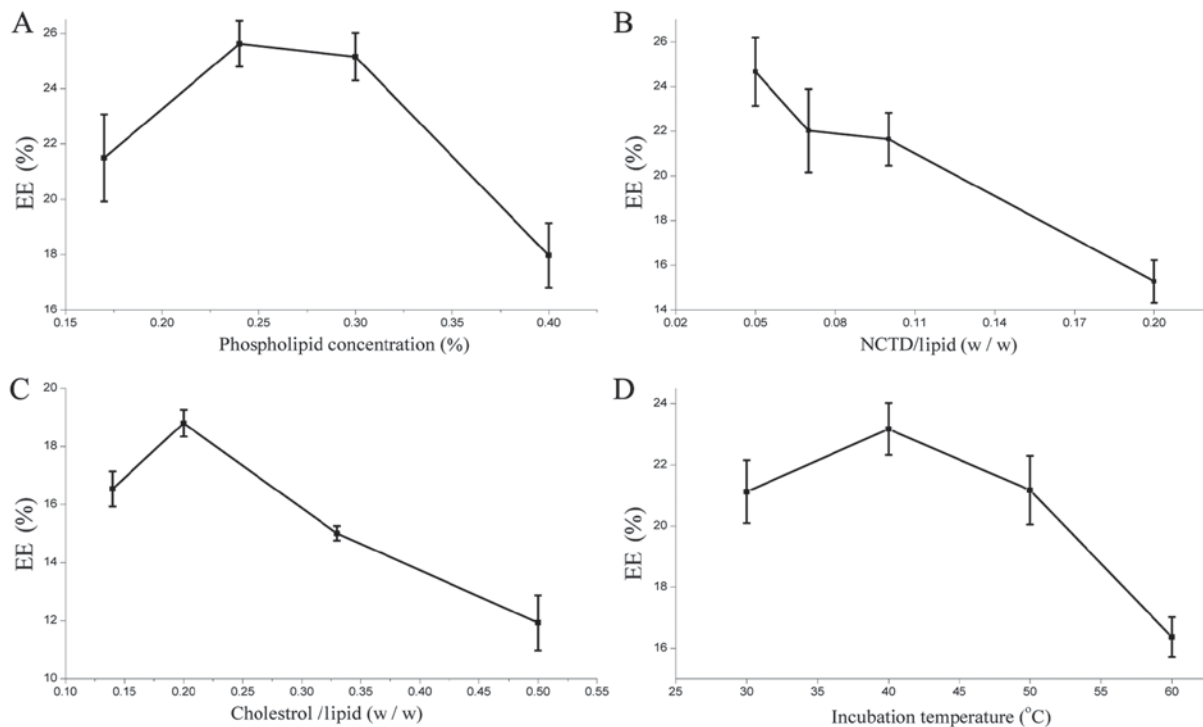


Figure 4. Effect of different preparation variables on the EE of SG-NCTD-LIP. Effect of changes in (A) phospholipid concentration (static parameters: 1:5 NCTD-phospholipid mass ratio and 1:7 cholesterol-phospholipid mass ratio at an incubation temperature of 50°C), (B) NCTD-phospholipid mass ratio (static parameters: 0.24% Phospholipid concentration, 1:7 cholesterol-phospholipid mass ratio at an incubation temperature of 50°C), (C) cholesterol-phospholipid mass ratio (static parameters: 0.24% Phospholipid concentration, 1:20 NCTD-phospholipid mass ratio at an incubation temperature of 50°C) and (D) incubation temperature (static parameters: 0.24% Phospholipid concentration, 1:20 NCTD-phospholipid mass ratio and 1:5 cholesterol-phospholipid mass ratio) on EE. EE, encapsulation efficiency; NCTD, norcantharidin.

A1B1C2D3, with 1:5 NCTD-phospholipid mass ratio, 0.4% phospholipid and 1:7 cholesterol-phospholipid mass ratio at an incubation temperature of 50°C. The analysis of variance indicated that factors A, B and C had a significant impact on the experimental outcome (Table III). According to the F-value, the impact of the factors may be scored as A>B>C>D, which supports earlier findings. Preparation and analysis of the optimal formulation mentioned above were repeated three times and the EE was 27.80±2.18%. Prior to optimization of the NCTD-LIP formulation, literature was consulted (41-44) and combined with preliminary experiments, which investigated the effect of different masses of SG on the size, morphology, EE and stability of liposomes. Following this, 0.04% SG was identified as an appropriate amount of drug to be added, without affecting the formulation parameters of the liposome.

SG-NCTD-LIP size and morphology. Nanoparticles, between 70-200 nm, function as drug delivery systems and are usually in the circulation for longer than free drugs (45,46). In addition, such nanoparticles escape RES clearance, while larger particles (>200 nm) may be removed by the system. A small particle size (<200 nm), below the pore size of leaky vasculatures, along with poor lymphatic drainage may provide a suitable condition for the accumulation and localization in the tumor (47). The average particle size at the optimal formulation after 3 months of storage at 4°C was 87.5 nm with a PDI of 0.151 (Fig. 5). The morphological character of SG-NCTD-LIPs was evaluated by TEM. Liposomes were spherical, no aggregation or fusion occurred and the size was ~100 nm.

Stability of liposomes SG-NCTD-LIPs. Particle size and EE were evaluated following 3 months of storage at 4°C to study stability. Initially, the average particle size was 87.5 nm and EE was 27.80%. There were no significant changes in the average particle size and EE following 3 months at 4°C, the average particle size was 82.9 nm and EE was 27.28%. The SG-NCTD-LIP stability was attributed to the presence of cholesterol, which increased the rigidity of the liposomal membrane and reduced permeability (48). The results demonstrated that SG-NCTD-LIPs were stable at 4°C for 3 months. However, the biological efficacy of SG-NCTD-LIPs following storage was not evaluated.

In vitro release of NCTD from SG-NCTD-LIP. The *in vitro* release rate evaluation describes an essential step for drug development and quality control, which may reflect on the *in vivo* drug performance. Q is used to denote the cumulative release percentage. The release rate of NCTD from SG-NCTD-LIP was significantly decreased compared with the free NCTD solution 1.5-12 h after incubation (Fig. 6). SG-NCTD-LIP may prolong the release of NCTD from liposomes. The release process of NCTD from SG-NCTD-LIP may be divided into two phases: In the first 4 h, the release rate was high, with 65% of the total dose released. Unencapsulated NCTD and NCTD coordinating to the surface of the lipid bilayer may be responsible for the rapid release. This phase was defined as a rapid release phase. It was followed by a steady release, known as the slow release phase. To investigate the release properties of SG-NCTD-LIP, release data were fitted using various release models, including zero-order, first-order, Higuchi (49) and Weibull (50) kinetic models, and the

Table I. Factors and levels for optimization using orthogonal design.

Level	Factor			
	A	B (%)	C (°C)	D
1	1:5	0.040	40	1:3
2	1:15	0.025	50	1:5
3	1:30	0.015	60	1:7

A, NCTD-phospholipid mass ratio; B, phospholipid concentration; C, incubation temperature; D, cholesterol-phospholipid mass ratio.

Table II. Results of orthogonal testing.

No.	A	B (%)	C (°C)	D	EE (%)
1	1.00	1.00	1.00	1.00	25.88
2	1.00	2.00	2.00	2.00	23.40
3	1.00	3.00	3.00	3.00	22.65
4	2.00	1.00	2.00	3.00	27.63
5	2.00	2.00	3.00	1.00	20.26
6	2.00	3.00	1.00	2.00	23.96
7	3.00	1.00	3.00	2.00	17.20
8	3.00	2.00	1.00	3.00	15.53
9	3.00	3.00	2.00	1.00	17.72
<i>K</i> ₁	71.93	70.71	65.37	63.86	N/A
<i>K</i> ₂	71.85	59.19	68.75	64.56	N/A
<i>K</i> ₃	50.45	64.33	60.11	65.81	N/A
<i>R</i>	21.48	11.52	8.64	1.95	N/A

A, NCTD-phospholipid mass ratio; B, phospholipid concentration; C, incubation temperature; D, cholesterol-phospholipid mass ratio; EE, encapsulation efficiency; *K_n*, sum of EE values at level *n*; *R*=*K_{max}*-*K_{min}*.

optimal fit was determined by correlation coefficient. The fitting equations of the release curves are presented in Table IV. The results indicated that the release from SG-NCTD-LIP follows a first order model.

In vitro cytotoxicity study. The cytotoxicity of SG-LIP, free NCTD, NCTD-LIP and SG-NCTD-LIP towards HepG2 cells was evaluated using the MTT cytotoxicity assay. SG-LIP was assessed as a control and the concentration of SG in SG-LIP was equal to its concentration in SG-NCTD-LIP. Following incubation for 48 h, an IR value of ~6.35% was determined for SG-LIP, indicating no significant cytotoxicity against HepG2 cells (data not shown). The inhibitory effect of the samples increased with the NCTD concentration; all treatments revealed dose-dependent cytotoxic activity (Fig. 7). NCTD-LIP and SG-NCTD-LIP demonstrated significantly increased toxicity compared with free NCTD. In addition, when NCTD concentration was 5, 10 and 20 µg/ml, SG-NCTD-LIP significantly increased cytotoxicity compared with NCTD-LIP after 48 h incubation. The IC₅₀ of SG-NCTD-LIP, NCTD-LIP and free NCTD were

Table III. Variance analysis.

Source	<i>S</i>	<i>f</i>	<i>MS</i>	<i>F</i> -statistic	P-value
A	102.15	2	51.08	157.02	<0.01
B	22.20	2	11.10	34.13	<0.05
C	12.64	2	6.32	19.43	<0.05
D	0.65	2	0.33	1.00	>0.05
Error	0.65	2	0.33	N/A	N/A

S, sum of deviation square; *f*, degree of freedom; *MS*, mean square; A, NCTD-phospholipid mass ratio; B, phospholipid concentration; C, incubation temperature; D, cholesterol-phospholipid mass ratio.

Table IV. Release curve-fitting equations of norcantharidin loaded liposomes modified with stearyl glycerylphosphatidylcholine.

Model	Fitting equation	<i>R</i>
Zero-order	$Q=0.0972t+0.2303$	0.9190
First order	$\ln(1-Q)=-0.3336t-0.2110$	0.9902
Higuchi	$Q=0.3732t^{1/2}+0.0350$	0.9764
Weibull	$\ln(1/1-Q)=0.6040 \ln t+0.7481$	0.9561

R, correlation coefficient; *Q*, accumulative release rate; *t*, time.

16.93, 24.03 and 49.79 µg/ml, respectively. SG-NCTD-LIP was 1.42-fold more cytotoxic compared with NCTD-LIP. SG modification increased the cytotoxicity of the liposomes, which may be associated with the interaction between SG exposed on the liposome surface and GA receptors on the cell membrane.

Conclusions. SG-NCTD-LIP was successfully prepared using the ethanol injection method and the determined properties, including particle size, EE, release profile and stability, may meet the potential requirements of liver cancer therapy based on the *in vitro* experiments, which overcome the limitations of conventional chemotherapy by improving the bioavailability and stability of the drug molecules, and minimizing side effects by site-specific and targeted delivery of the drugs (51-53). The *in vitro* cytotoxicity study confirmed that SG modification of NCTD-LIP enhanced the inhibitory effects on hepatoblastoma cells. Therefore, SG-NCTD-LIP may be a potential drug delivery system for NCTD-targeted liver cancer therapy. Future experiments may include cytotoxicity studies against HepG2 cells compared with L02 normal liver cells, to confirm the safety and efficacy of SG-NCTD-LIP, further uptake studies and *in vivo* experiments.

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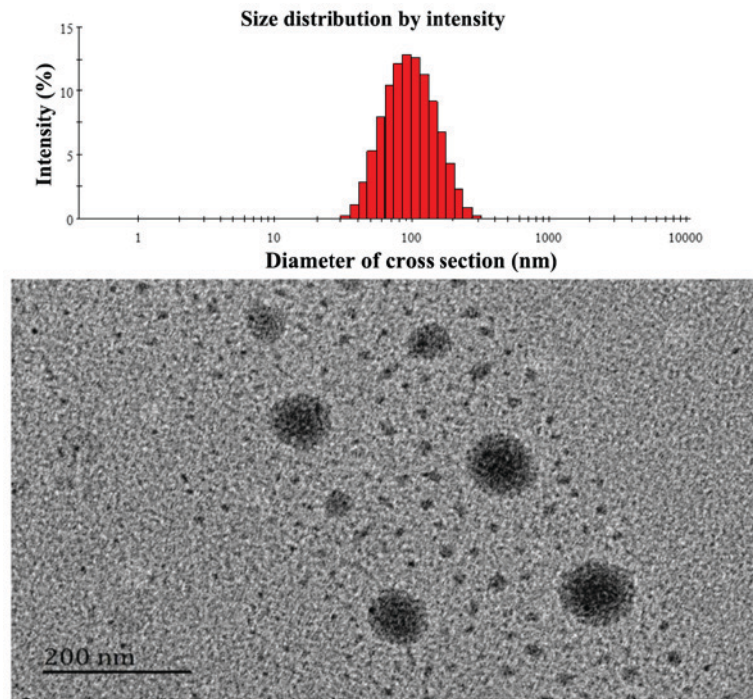


Figure 5. Particle size distribution (intensity) and TEM image of norcantharidin-loaded liposomes modified with stearyl glycyrrhetinate. TEM, transmission electron microscopy.

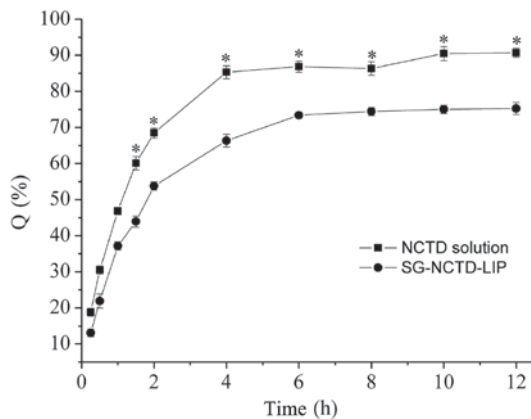


Figure 6. *In vitro* release of NCTD from solution and SG-NCTD-LIP over time. NCTD, norcantharidin; SG-NCTD-LIP, NCTD-loaded liposomes modified with stearyl glycyrrhetinate; Q, cumulative release percentage. * $P < 0.05$ vs. SG-NCTD-LIP.

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Availability of data and materials

The datasets generated and analyzed during the current study are not publicly available due to the patent application but are available from the corresponding author on reasonable request.

Authors' contributions

WW was involved in the design of the study. JZ and WZ performed all experiments. DW performed the *in vitro* cytotoxicity studies. SL performed statistical analyses. All authors

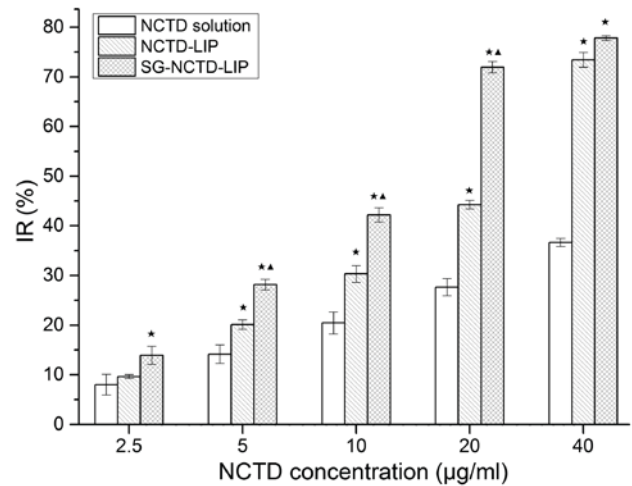


Figure 7. Inhibitory effects of different concentrations of NCTD in solution, NCTD-LIP and SG-NCTD-LIP on HepG2 cells after 48 h. * $P < 0.05$ vs. NCTD solution; $\Delta P < 0.05$ vs. NCTD-LIP. NCTD, norcantharidin; LIP, liposome; NCTD-LIP, NCTD-loaded liposomes; SG-NCTD-LIP, NCTD-loaded LIPs modified with stearyl glycyrrhetinate; IR, inhibition ratio.

participated in manuscript preparation and involved in the discussion of the results. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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