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Lyophilized Cheliensisin A submicron emulsion for intravenous injection: Characterization, *in vitro* and *in vivo* antitumor effect

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Abstract

Growing attentions have been focused on natural antitumor drugs. Recently, a novel and potent antitumor drug Cheliensisin A (GC-51) with broad-spectrum efficiency has been developed. However, due to its poor water solubility and chemical instability, choosing the appropriate dosage form is of great significance. This study aimed at developing a lyophilized submicron emulsion for GC-51 and further improving the therapeutic index of the drug. The resultant lyophilized GC-51 submicron emulsion was much more stable than its solution, which can be stored for years without significant change on physicochemical properties. And its solubility was increased from 6.74 ± 0.14 to 2.00 ± 0.10 mg mL^{-1}. The 50% inhibitory concentration IC_{50} values were calculated from growth curves by MTT assay on various tumor cell lines. Compared with the IC_{50} of GC-51 crude drug, that of lyophilized GC-51 submicron emulsion decreased from 24.04 ± 1.97 to 8.23 ± 1.84 μg mL^{-1} on HepG2, and from 31.08 ± 2.56 to 10.85 ± 2.09 μg mL^{-1} on CT-26, from 17.90 ± 1.83 to 7.49 ± 1.87 μg mL^{-1} on HeLa and from 16.38 ± 2.41 to 10.13 ± 2.12 μg mL^{-1} on A549, respectively. In the time-dependent assay of tumor cell viability, lyophilized GC-51 submicron emulsion exhibited significantly lower inhibition rate in the initial action times, but increased gradually afterwards. That means lyophilized submicron emulsion as the vector for GC-51 had some protective and delayed release effect. Further, the *in vivo* therapeutic efficacy was measured in pulmonary metastasis of colon cancer-bearing BALB/c mice model. An obvious enhanced antitumor activity was observed after administration of lyophilized GC-51 submicron emulsion ($P < 0.05$), which increased from 22.78 ± 3.5 to 41.42 ± 4.2% compared with GC-51 injection. And the life span of tumor-bearing mice in lyophilized GC-51 submicron emulsion group was significantly longer than that of the mice in GC-51 injection and normal saline groups. Compared with crude drug, the lyophilized GC-51 submicron emulsion showed a significantly higher antitumor efficiency both *in vivo* and *in vitro*, suggesting a potential application in tumor chemotherapy.

Keywords: Cheliensisin A; Lyophilized submicron emulsion; Antitumor drug; Solubility; Stability; *In vivo* and *in vitro* antitumor efficiency

1. Introduction

Cheliensisin A (GC-51, 6(7,8-epoxy-styryl)-5-acetoxy-5,6-dihydro-2-pyrone) is a natural crystal compound extracted from *Goniothalamus cheliensis Hu* in Bull, Goniothalamus, Annonaceae (Fig. 1). *In vitro* study suggests that GC-51 possesses a broad-spectrum efficiency, including inducing apoptosis, activation of caspase-3 and downregulation of Bcl-2 mRNA expression (Zhong et al., 2005). However, its application has been strongly impeded due to its easy hydrolysis and poor water solubility. Intravenous injection might be a suitable way for administration to deliver the drug and maximize its antitumor efficiency.

According to literature, O/W submicron emulsion has been introduced as an effective delivery vehicle for non-polar drugs whose intravenous administration is greatly hampered by their low solubility in water (Georges et al., 2003; Narin et al., 2006; Wim et al., 2006). In addition, formulations of intravenous emulsions, such as diazepam, propofol, fat soluble vitamins or amphotericin B, have already been developed to access to market. This formulation is also appealing to ocular, cutaneous or oral drug delivery (Klang et al., 1996; Ilan et al., 1996; Sznitowska et al., 1994; Dollo et al., 2003). However, a major problem of the lipid submicron emulsion is the physical–chemical instability during long-term storage. One possible way to improve its stability is to formulate it

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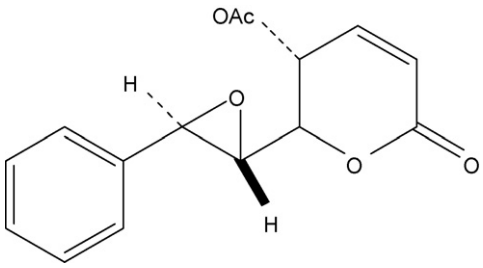

Fig. 1. Chemical structure of Cheliensisin A (GC-51).

into dry emulsions (Christensen et al., 2002; Takeuchi et al., 1992; Molina and Cadorniga, 1995; Myers and Shively, 1992). Moreover, the content of GC-51 in submicron emulsion was found to decrease gradually in aqueous environment, which may result from the hydrolyzation effect. Typically, dry emulsion formulations are prepared from O/W emulsions containing a soluble or an insoluble solid carrier in the aqueous phase by spray drying (Christensen et al., 2002; Takeuchi et al., 1992; Molina and Cadorniga, 1995), lyophilization (Myers and Shively, 1992) or evaporation (Carmichael et al., 1987). Dry emulsions are regarded as lipid-based powder formations from which an O/W emulsion can be reconstituted. From a pharmaceutical point of view, dry emulsion formulations are attractive in this case due to their physical advantages and ease of administration as capsules, tablets, and lyophilized sterile powders.

In this study, we developed a unique lyophilized submicron emulsion which improved the water solubility and chemical stability of GC-51. The antitumor efficacy of lyophilized GC-51 submicron emulsion was evaluated by studying its *in vitro* cytotoxicity and *in vivo* therapeutic effects in pulmonary metastasis of colon cancer in BALB/c mice model. Compared with its crude drug, lyophilized GC-51 submicron emulsion showed a significantly higher antitumor efficiency both *in vivo* and *in vitro*, suggesting a potential application in tumor chemotherapy.

2. Materials and methods

2.1. Materials

GC-51 was provided by Kunming Institute of Botany, Chinese Academy of Science. Medium chain triglycerides (EP) were purchased from GATTEFOSSE SAS (France). Soybean lecithin and vitamin E were provided by Shanghai Taiwei Co. (China). Sucrose was obtained from Guanghua Chemical Factory, Shantou (China). Double-distilled water was used for all solutions and dilution. RPMI-1640 and DMEM cell culture medium were purchased from Gibco (Invitrogen Corporation, Grand Island, NY). Calf serum was obtained from Lanzhou Minghai Biological Company (China). GC-51 injection was prepared in the following way: GC-51 was dissolved in the mixed solvents, consisting of cremophor:ethanol:PBS (1:1:5, v/v/v). All the other chemicals and solvents used in this study were of analytical grade.

2.2. Physicochemical properties of GC-51

2.2.1. Solubility

Excess amount of GC-51 was placed in three microtubes which contained 1.0 mL deionized water, 1.0 mL 0.9% NaCl and 1.0 mL 1.0% DMSO, respectively and shaken at 25 °C, 60 rpm for 12 h to ensure the solubility equilibrium. At the end of this step, solution was centrifuged at 12,500 rpm for 10 min and the supernatant was filtered through 0.45 μm filter membrane (Millipore). And 20 μL of the filtered supernatant was analyzed by HPLC.

2.2.2. Apparent partition coefficient

Accurately weighed amounts of GC-51 were partitioned between double-distilled water and *n*-octanol for 12 h at room temperature by the shake flask method. GC-51 concentrations in the aqueous phase before and after partitioning were measured through HPLC analysis, respectively, and the partition coefficients from the results were calculated as $\log P = \log(C_0 - C_{aq})/C_{aq}$.

2.2.3. HPLC analysis

The HPLC system consisted of a Waters 2690 separation module and a 996 Photodiode Array (PDA) detector; and data were collected and processed using Millennium software Version 3.2 (all equipments from Waters, Milford, MA, USA). Chromatographic separations were carried out on SepaxGP-C_{18} (Sepax Technologies Inc., USA) reverse phase column, VP-ODS (150 mm × 4.6 mm, 5 μm) equipped with a Shimadzu Shimpack G guard column (C_{18}, 10 mm × 4 mm, 5 μm) (Chiyoda-Ku, Tokyo, Japan). The mobile phase was consisted of water and acetonitrile (60:40, v/v), newly prepared daily and filtered through a 0.22-μm membrane filter and degassed via an online degasser. The injection volume was 20 μl each and eluted at a flow rate of 1.0 mL min^{-1} at 35 °C. The eluents were monitored at 192 nm (AUFS = 1). The analytical method was proved to be valid with respect to precision (R.S.D. = 0.87%), linearity ($r^2 = 0.9998$), reproducibility (R.S.D. = 1.24%) and selectivity (no interfering peaks were observed).

2.3. Preparation and characterization of lyophilized GC-51 submicron emulsion

2.3.1. Preparation of lyophilized GC-51 submicron emulsion

The aqueous phase (600 mL) and oil phase (200 g) were separately prepared. 2.0 g GC-51, 40.0 g soybean lecithin and 0.3 g vitamin E were dissolved in 200.0 g medium chain triglycerides. Both of the phases were heated separately to 70 °C, after which the two phases were mixed and sheared by a high shear mixer (A-88, Jingtan Medical Instrument Factory, Jiangsu, China) for 5 min and rapidly cooled to 20 °C below. And then the emulsion was further homogenized using high-pressure homogenizer (EmulsiFlex-C5, AVESTIN, Canada) at 200 kg cm^{-2} for six times, and diluted to 1000 mL with double-distilled water. Sucrose (10%, w/v) was dissolved in the emulsion as cryoprotection agent. Subsequently, the homogenized emulsion was

filtrated through a 0.80-μm filter membrane (Millipore, USA) to vials, pre-frozen for 4 h at −40 °C, lyophilized for 24 h. Then, the resultant lyophilized GC-51 submicron emulsion was exposed to 25kGy γ-rays using ^{60}Co (FJx424, China's second Institute for Nuclear Physics) as the radiation source (Tsai et al., 1986). The temperature was 18–21 °C, and the humidity was 60–70%. (When the experiment is up-scaled, it can be sterilized by aseptic manufacture procedure.)

2.3.2. Particle size and zeta potential analysis

The mean particle size (*z*-average) and zeta potential of the lipid emulsions were determined by photon correlation spectroscopy (Malvern Nano ZS90, UK). A 1:100 dilution of the emulsions was prepared with double-distilled water before each measurement.

2.3.3. Transmission electron microscopy (TEM)

The morphology of the lyophilized GC-51 submicron emulsion was examined by TEM (H-600, Hitachi, Japan). Before analysis, the samples were diluted 1:2 and stained with 2% (w/v) phosphotungstic acid for 30 s and placed on copper grids with films for observation.

2.4. Stability test

The resultant lyophilized GC-51submicron emulsions were stored at 25 °C for 12 months. The average size, PDI, zeta potential and drug contents were determined at predetermined time intervals. Each sample was detected for three times.

In addition, redispersed lyophilized GC-51 submicron emulsion by double-distilled water and GC-51 injection were placed on the shaking bed at 25 and 37 °C, respectively. Drug content was determined on days 0, 0.5, 1–7, which aimed at investigating the stabilizing effect of the emulsion.

2.5. In vitro cytotoxicity

HepG2, CT-26, A549 and HeLa cells, obtained from Institute of Biochemistry and Cell Biology, Institutes for Biological Sciences, Chinese Academy of Science (Shanghai), were cultured with RPMI-1640 or DMEM medium (Gibco, USA) supplemented with 10% bovine serum, 100 U mL^{-1} penicillin and streptomycin (100 μg mL^{-1}).

100 μl of single cell suspension was seeded to 96-well plate at the concentration of 1×10^4 cells per well. Blank lyophilized submicron emulsion, lyophilized GC-51 submicron emulsion and GC-51 crude drug (dissolved in RPMI-1640 or DMEM medium containing 1.0% DMSO) were diluted with culture medium to 0.1 mg mL^{-1} as stock solution, respectively. All the experimental groups were pre-sterilized. MTT assays were carried out at a series of drug concentrations: 1, 5, 10, 20, 40, and 80 μg mL^{-1}(Grever et al., 1992). After a 24-h exposure, all medium was discarded and the wells were rinsed with PBS twice. 100 μl of fresh culture medium was added into each well. Subsequently, 20 μl of MTT (5 mg mL^{-1}) was added and the plate was incubated to produce formazan crystals. 4 h later, the formazan crystal was dissolved in 150 μl of DMSO (Sigma, USA). The quantity of formazan products was measured at 570 nm using a microplate reader (Bio-Rad Model 550, USA). The cell viability (%) related to control wells containing cell culture medium was calculated by the following equation:

$$\text{cell viability percentage}\,(\%) = \frac{[A]_{\text{test}} - [A]_0}{[A]_{\text{control}} - [A]_0} \times 100$$

Cytotoxicity was expressed by the concentration causing a 50% inhibition of cell proliferation (IC_{50}) (Wang et al., 2006). When the cell viability percentage of GC-51 crude drug was estimated, $[A]_{\text{control}}$ was interpreted as the absorption value (A) of the wells cultured with medium containing 1% DMSO.

Meanwhile, a time-dependent assay was conducted with a predetermined concentration of 40 μg mL^{-1} for each well at 1, 2, 4, 8, 12, and 24 h, respectively. Similarly, the cell viabilities under different time intervals were determined by MTT assay as mentioned above.

2.6. In vivo therapeutic effect

2.6.1. Cells

BALB/c-derived, CT-26 colon adenocarcinoma cell line was grown in RPMI-1640 supplemented with 10% FCS, 0.56% $NaHCO_3$, 100 U mL^{-1} penicillin, and 0.1 mg mL^{-1} streptomycin. Cultures were maintained in a humidified 5% CO_2 atmosphere at 37 °C.

2.6.2. Animal and tumor model

72 BALB/c mice (half male and half female; body weight, 18–22 g), purchased from West China Experimental Animal Center of Sichuan University (China), were fed in a germ-free environment with free access to food and water. CT-26 cells were prepared as single cell suspension in sterile PBS at a concentration of 2.5×10^6 mL^{-1}, and a volume of 200 μl of cell suspension was injected into the lateral tail vein of BALB/c mice. All animal experiments were performed according to our institutional and NIH guidelines for care and use of research animals.

2.6.3. Grouping

Mice were randomly divided into eight groups, with eight mice each group. Among them, Groups 2–8 were pre-injected with tumor cells. Twenty-four hours later, different doses (5, 15, 30 mg kg^{-1}) of GC-51 submicron emulsion were given via tail vein injection to the Groups 5–7, respectively, while GC-51 injection (30 mg kg^{-1}) was given to Group 8. Group 2 received normal saline as negative control, Group 3 received the blank solvent of GC-51 injection, Group 4 received blank emulsion, while Group 1 was treated as blank control without being injected with tumor cells nor drug formulations.

The experiment was conducted by administering testing samples on days 1, 7 and 14, respectively. On the 15th day after inoculation, all mice were sacrificed, leaving the organs to be dissected, weighed and compared. The tumor inhibition rate was

Table 1
Solubility values and log *P* of GC-51

Test compound	GC-51
Solubility ($\mu g\,mL^{-1}$)	
Deionized water	6.74 ± 0.14
0.9% NaCl	7.02 ± 0.08
1.0% DMSO	95.42 ± 2.10
log *P*	1.98 ± 0.05

Each value represents the mean ± S.D. ($n = 3$).

calculated by the equations given below:

$$A_{[\text{organ index}]} = \frac{W_{[\text{organ}]}}{W_{[\text{animal}]}} \times 1000$$

Here $W_{[\text{organ}]}$ refers to the weight of a specific organ; $W_{[\text{animal}]}$ refers to the total body weight of the animal.

$$\text{tumor inhibition rate}\,(\%) = \left(1 - \frac{A_1 - A_0}{A_2 - A_0}\right) \times 100$$

Here A_1, A_2, A_0, refers to lung index of therapeutic group, lung index of negative control group and lung index of blank control group, respectively. However, when calculated the tumor inhibition rate of GC-51 crude drug, A_2 refers to the organ index of the blank solvents of the GC-51 injection.

2.6.4. Histological examination

The lung tissue samples were fixed in 10% formalin buffer and embedded in paraffin. Five-micrometer thick sections were placed on polylysine-coated slides and stained with hematoxylin and eosin (H&E). The stained slides were evaluated under inverted microscope (Axiovert 40 CFL, Carl Zeiss, Oberkochem, Germany) at 100× magnification and were digitally scanned and the images were enhanced using a computer software system (AxioVision Release 4.1, Carl Zeiss, Oberkochem, Germany) for histological examination.

2.6.5. Survival curve

Lung cancer model was established in 70 BALB/c mice as described above. BALB/c mice were randomly divided into 7 groups, with 10 mice in each group. The experiment was conducted by administering testing samples on days 1, 7 and 14, respectively.

2.7. Statistical analysis

Particle size, PDI, zeta potentials, *in vitro* cytotoxicity and *in vivo* antitumor effect studies were performed in triplicate unless specified. The data are illustrated as mean *F* standard deviation (S.D.). Data were analyzed using the Student's *t*-test and one-way analysis of variance (ANOVA) ($P < 0.05$).

3. Results and discussion

3.1. Solubility and log P

Table 1 summarizes the solubility values of GC-51 in different solvents, which shows GC-51 has a poor solubility in aqueous solution. However, 1.0% DMSO can greatly improve its solubility. Besides, the log *P* values for GC-51 was also shown.

3.2. Preparation and characterization of GC-51 submicron emulsion

The resultant emulsion mainly consisted 4.0% (w/v) soybean lecithin, 20.0% (v/v) medium chain triglycerides and 80.0% (v/v) double-distilled water. The content of GC-51 in the submicron emulsion was $2.0\,mg\,mL^{-1}$, which was much higher than that in its saturable water solution. The average size of the emulsion was in the range of 100–200 nm. Table 2 shows the average particle size, PDI, and zeta potential of the GC-51 submicron emulsions prepared in this study. The particle size (*z*-average) of the freshly prepared emulsion was 153.4 ± 5.2 nm (mean ± S.D.), which could be characterized as a submicron level. The PDI could also be well-controlled to a narrow range of less than 0.2. The zeta potential is around −35.3 to −28.2 mV as shown in Table 2. The anionic fractions such as phosphatidylserine, phosphatidic acid, phosphatidylglycerol and phosphatidylinositol in soybean lecithin were responsible for the negative zeta potential. Since they had a surface charge of −35.3 to −28.2 mV, the droplets were charge-stabilized colloids. And no significant difference was observed between freshly prepared lyophilized GC-51 submicron emulsion and lyophilized GC-51 submicron emulsion which had been sterilized by ^{60}Co γ-ray in terms of particle size, PDI, zeta potential and drug content as shown in Table 2. The appearance of the lyophilized GC-51 submicron emulsion was porosity and clumping. After being sterilized by ^{60}Co γ-ray, we could find that there were no significant change on both appearance and morphology of lyophilized GC-51 submicron (Fig. 2).

TEM image of lyophilized GC-51 submicron emulsion is presented in Fig. 2. This result confirms that the emulsions are circular in shape with diameters of 100–300 nm.

Generally, it is considered that the formulation of submicron emulsion is a kind of hydrodynamically unstable system. Due to this inherent physicochemical instability, emulsions may easily aggregate to creaming, flocculation and experience a phase inversion during long-term storage. In the liquid form, the phospholipids are easy to be oxygenized and degraded. Therefore, submicron emulsions for injection have relatively a short shelf-life. Surprisingly, the lyophilized submicron emulsion, with a physicochemical stability could prolong shelf-life to an advanced level. With respect to lyophilization process, water molecules in the dispersion phase were removed via sublimation, and they rehydrated easily and quickly because of the porous structure left after the ice has sublimed.

Table 2
The average particle size and zeta potential characterization of GC-51 submicron emulsions with medium chain triglycerides as oil phase before and after lyophilization, and after radio-sterilization

Items	Average size (nm)	Polydispersity index	Zeta potential (mV)	Drug content (%)
Plain emulsion	125.5 ± 3.1	0.119 ± 0.014	−30.6 ± 2.4	–
GC-51 submicron emulsion before lyophilization	153.4 ± 5.2	0.082 ± 0.025	−31.5 ± 3.2	99.5 ± 0.2
GC-51 submicron emulsion after lyophilization	169.5 ± 4.1	0.119 ± 0.017	−31.7 ± 1.3	99.4 ± 0.2
Lyophilized GC-51 submicron emulsion after radio-sterilization	171.1 ± 5.2	0.123 ± 0.014	−32.5 ± 2.8	99.1 ± 0.3

Each value represents the mean ± S.D. ($n = 3$).

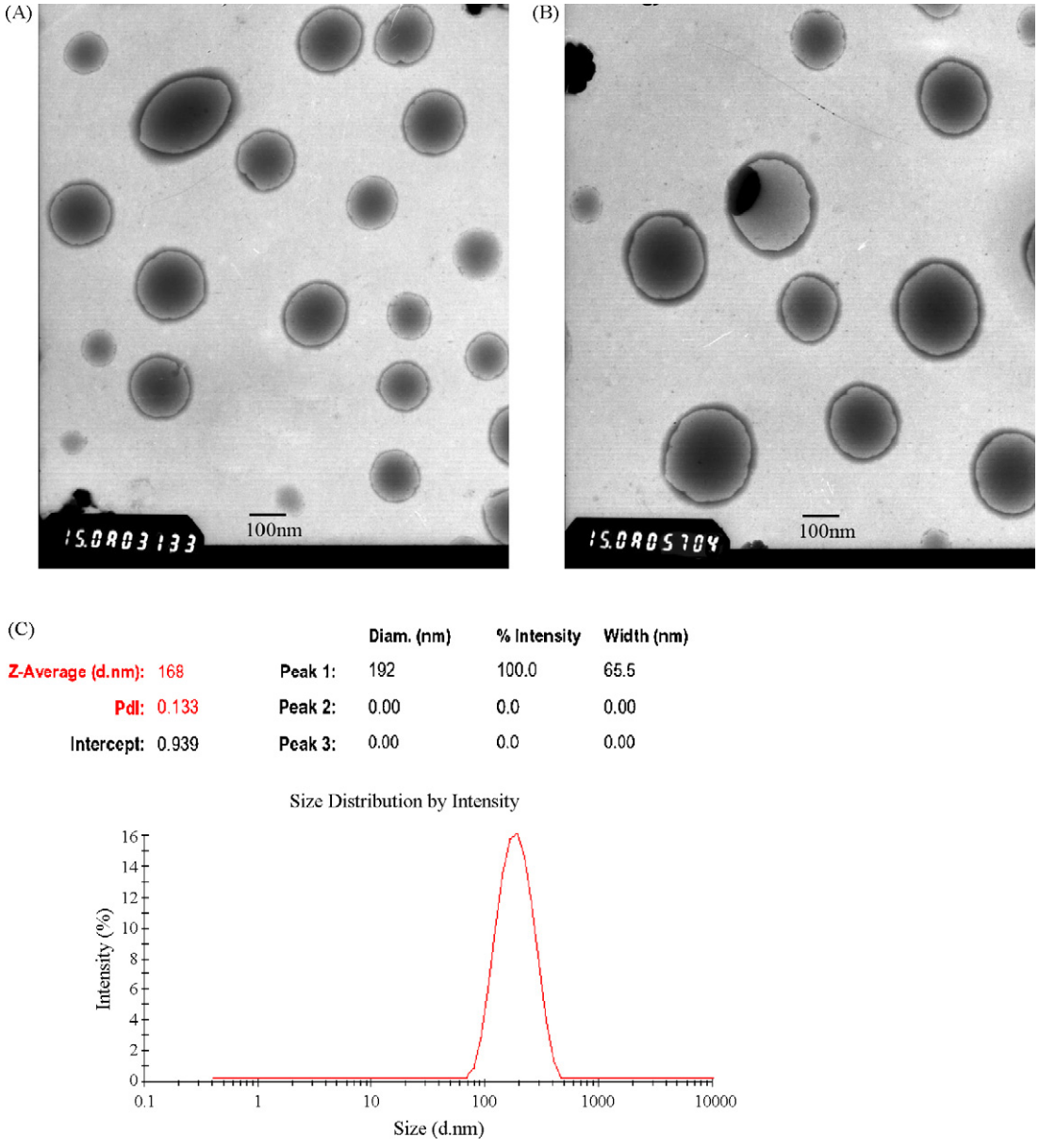

Fig. 2. Transmission electron micrograph of GC-51 submicron emulsion (A) and sterilized GC-51 submicron emulsion by ^{60}Co γ-ray (B), scale bar: 100 nm; and the size distribution of lyophilized GC-51 submicron emulsion, which was detected by photon correlation spectroscopy (Mlvern Nano ZS90, UK) (C).

3.3. Stability test

As shown in Table 3, the lyophilized GC-51 submicron emulsion was physically and chemically stable during long-term storage. And as shown in Fig. 3, when redispersed in water, lyophilized GC-51 submicron emulsion proved to be more stable than its crude drug solution, which indicated that the emulsion formulation had certain protective effect to GC-51, in accordance with *in vitro* antitumor assay as well. Therefore, it is inferred that the lyophilized GC-51 submicron emulsion may

Table 3
Lyophilized GC-51 submicron emulsion stored at 25 °C ($n = 3$)

Time (month)	Size (nm)	PDI	Zeta potential (mV)	Content (%)
0	169.4 ± 3.1	0.121 ± 0.015	−30.2 ± 1.2	99.4 ± 0.2
1	171.3 ± 3.5	0.122 ± 0.023	−32.2 ± 1.5	100.5 ± 0.2
3	172.1 ± 2.4	0.123 ± 0.025	−32.5 ± 1.7	98.7 ± 0.3
6	169.8 ± 4.5	0.121 ± 0.022	−30.5 ± 1.6	98.4 ± 0.3
12	183.4 ± 2.8	0.112 ± 0.016	−28.6 ± 1.4	98.2 ± 0.1

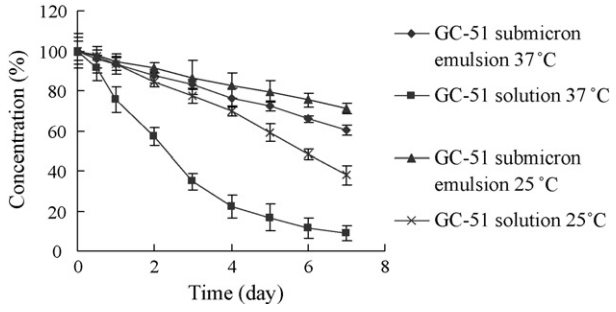

Fig. 3. Time course of stability of GC-51 submicron emulsion and GC-51 injection at 37 and 25 °C. Data are mean ± S.D. ($n = 3$).

have sustained release effect. And it is demonstrated that, the submicron emulsion is more stable at 25 °C than at 37 °C. So we can conclude that the higher temperature can increase the instability of aqueous solution of GC-51.

3.4. In vitro cytotoxicity

The cytotoxicity (IC_{50}) of GC-51 crude drug and lyophilized GC-51 submicron emulsion was shown in Table 4. Lyophilized GC-51 submicron emulsion showed significantly increased inhibition rate than GC-51 crude drug on HepG2, CT-26, A549 and HeLa cells. Compared with GC-51 crude drug, the IC_{50} of lyophilized GC-51 submicron emulsion decreased from 24.04 ± 1.97 to $8.23 \pm 1.84\ \mu g\ mL^{-1}$ on HepG2, and from 31.08 ± 2.56 to $10.85 \pm 2.09\ \mu g\ mL^{-1}$ on CT-26, from 17.90 ± 1.83 to $7.49 \pm 1.87\ \mu g\ mL^{-1}$ on HeLa and from 16.38 ± 2.41 to $10.13 \pm 2.12\ \mu g\ mL^{-1}$ on A549, respectively. All test groups showed a concentration-dependent manner of action (Figs. 4 and 5).

In addition, blank submicron emulsion exhibited low toxicity or even nontoxicity under various concentrations, indicated that the excipients in this formulation were low

Table 4
IC_{50} values of GC-51 crude drug and GC-51 submicron emulsion on different cell lines (24 h, $n = 3$)

Cell lines	IC_{50} ($\mu g\ mL^{-1}$)	
	GC-51 crude drug	GC-51 submicron emulsion
HepG2	24.04 ± 1.97	8.23 ± 1.84
CT-26	31.08 ± 2.56	10.85 ± 2.09
HeLa	17.90 ± 1.83	7.49 ± 1.87
A549	16.38 ± 2.41	10.13 ± 2.12

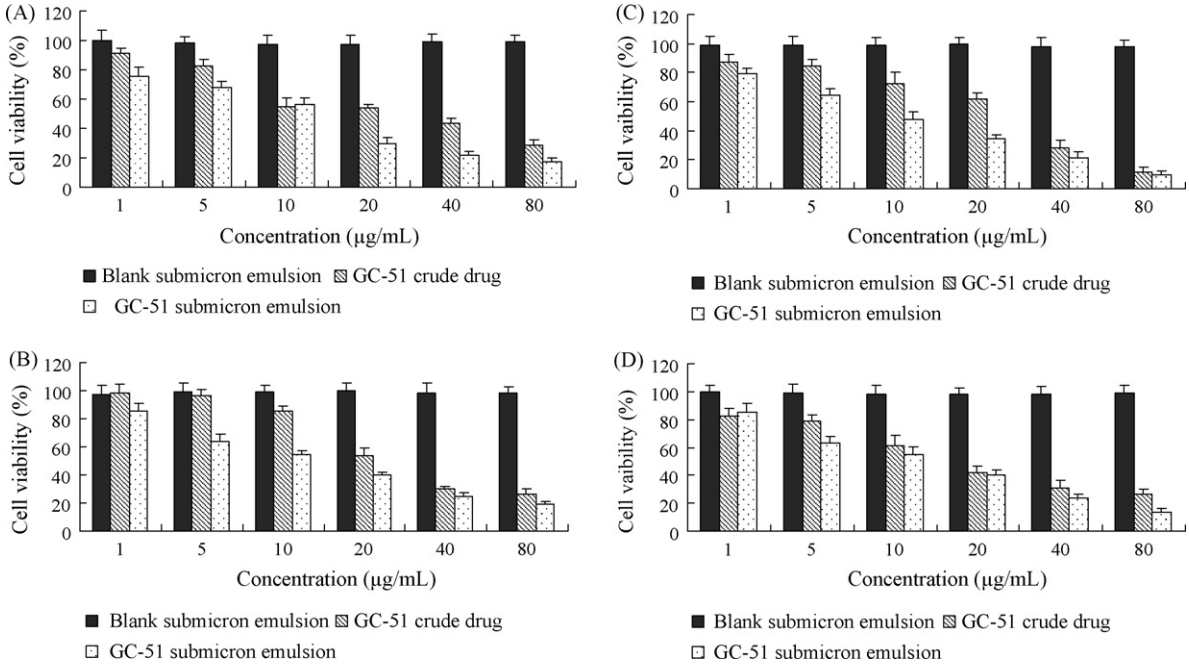

Fig. 4. Cell viability of HepG2 (A), CT-26 (B), HeLa (C) and A549 (D) cells after 24 h of treatment of GC-51 submicron emulsion, GC-51 crude drug and blank submicron emulsion ($n = 3$).

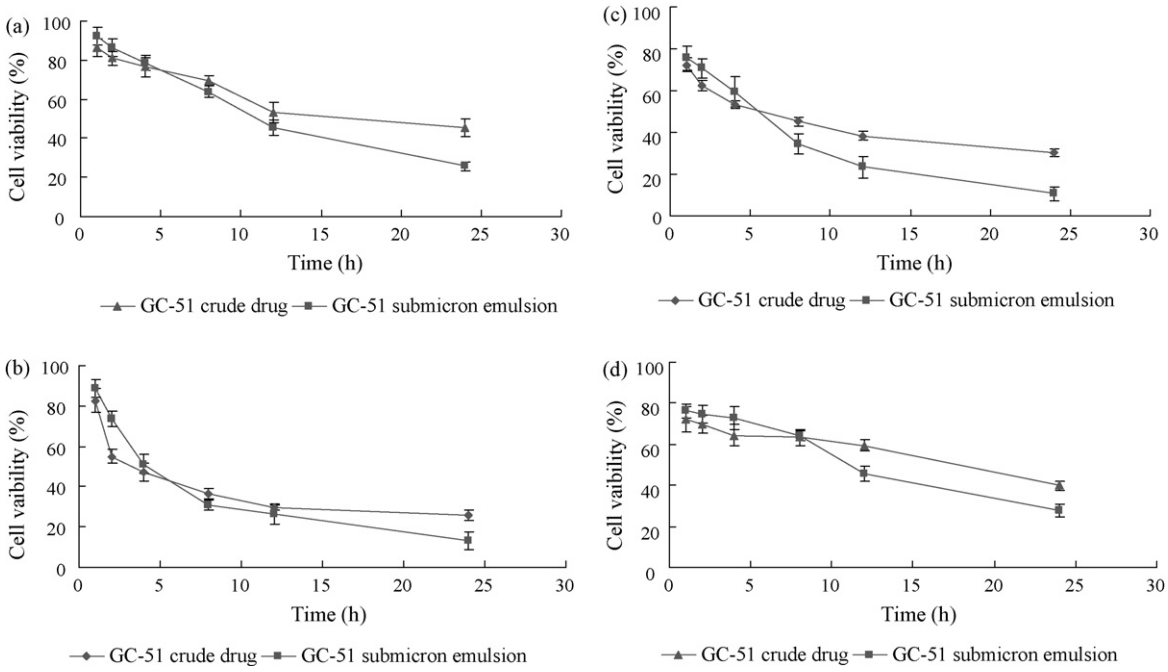

Fig. 5. Cell viability of HepG2 (a), CT-26 (b), HeLa (c) and A549 (d) cells after different action times of treatment of GC-51 crude drug and GC-51 submicron emulsion ($n = 3$).

toxic or even nontoxic under formulation concentrations. In previous study, it was well established that the cytotoxicity of drugs was strongly dependent on the type of treated cells, time of contact, etc. IC_{50} values are shown in Table 4.

The time-dependent assay of tumor cell viability percentage showed similar results on different tumor cell lines (Fig. 6). Compared with GC-51 crude drug, lyophilized GC-51 submicron emulsion exhibited significantly lower inhibition rate in the initial action times, but increased gradually afterwards. Considering the results of the stability assay above, it is inferred that the lyophilized submicron emulsion as the vector of GC-51 had certain protective and delayed release effect which resulted in the increased antitumor effect.

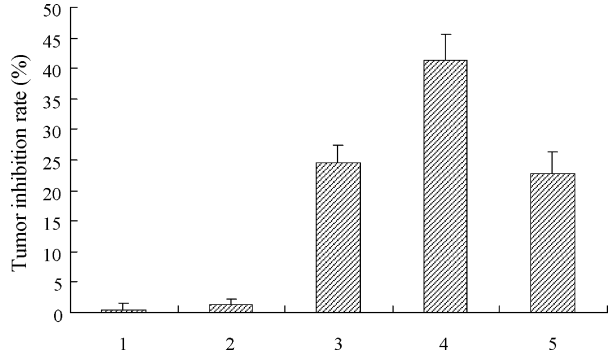

Fig. 6. Antitumor effect of GC-51 modalities administered into mice-bearing lung carcinoma by i.v. administration ($n = 8$); (1) blank submicron emulsion; (2–4) GC-51 submicron emulsion 5, 15, and 30 mg/kg; (5) GC-51 crude drug 30 mg/kg.

3.5. In vivo antitumor efficacy of GC-51 submicron emulsion

Compared with normal saline group, the therapeutic groups all showed certain tumor inhibition effect. Especially, lyophilized GC-51 submicron emulsion exhibited a concentration-dependent manner (Fig. 6) as well as an enhanced antitumor activity, compared with GC-51 injection ($P < 0.05$), which increased from 22.78 ± 3.5 to $41.42 \pm 4.2\%$. It was assumed that, as the vector, the lyophilized submicron emulsion delayed the release of GC-51 and increased its pharmacodynamic action.

3.6. Histological examination

HE stained micrograph (Fig. 7) showed that compared with normal lung tissue sections, the lung tissues of the physiological saline, blank submicron emulsion group and blank solvent of GC-51 injection group had severe tumor metastases, with intense condensation of cell nucleuses and the appearance of pulmonary fibrosis. With respect to therapeutic groups, lyophilized GC-51 submicron emulsion (30 mg/kg) and GC-51 injection groups (30 mg/kg) showed the least tumor metastases, which illustrated that the tumor growth in these two groups has been well controlled. And the middle and low dose-treated groups showed certain tumor inhibition effect compared with physiological saline group. In addition, the tumor inhibition effect of GC-51 injection was lower than that of lyophilized GC-51 submicron emulsion at the same administration dosage. Moreover, the *in vivo* tumor inhibition effect of lyophilized GC-51 submicron emulsion was concentration-dependent, which well corresponded with the result of *in vivo* antitumor study.

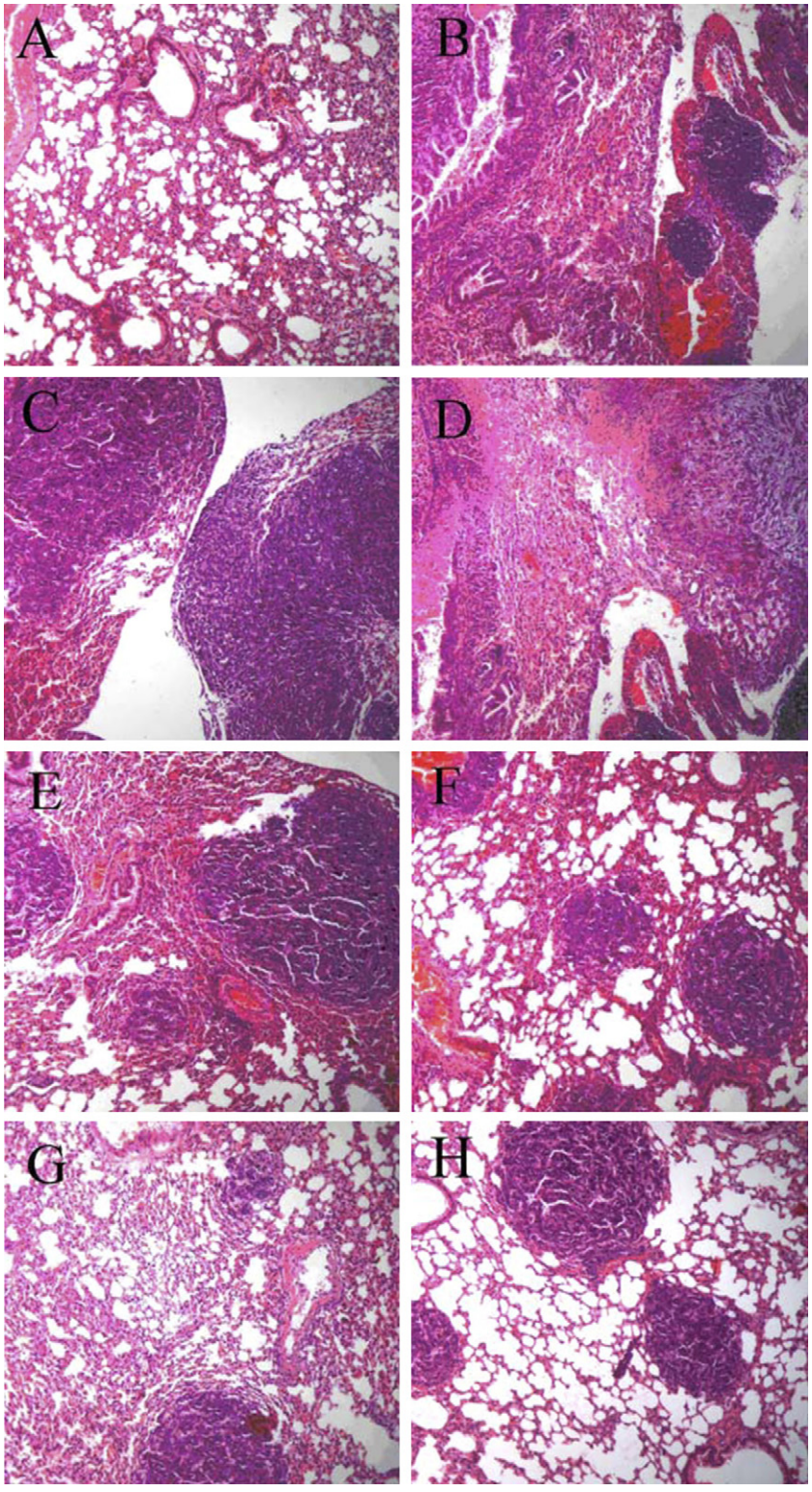

Fig. 7. Microphotographs of the hematoxylin–eosin stained BALB/c mice lung tissue sections (100× magnification); marks—(A) normal lung tissue; (B) physiological saline; (C) blank submicron emulsion; (D) blank solvent of GC-51 injection; (E) GC-51 submicron emulsion 5 mg/kg; (F) GC-51 submicron emulsion 15 mg/kg; (G) GC-51 submicron emulsion 30 mg/kg; (H) GC-51 crude drug 30 mg/kg.

3.7. Comparison of survival rate

We successfully established mice lung metastasis tumor models and examined the therapeutic efficacy of lyophilized GC-51 submicron emulsion and GC-51 injection. The experiment results showed that the life span of tumor-bearing mice in GC-51 injection and lyophilized GC-51 submicron emulsion groups were significantly longer than that of the mice in normal

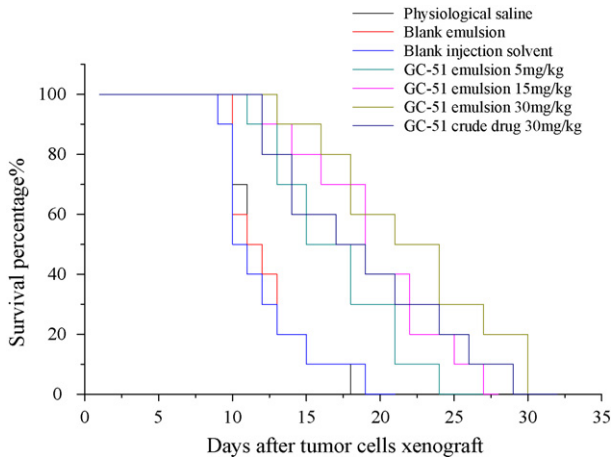

Fig. 8. Comparison of survival rates of BALB/c in different groups after therapy.

saline group, blank solvent of GC-51 injection group and blank emulsion group (Fig. 8). Similar with *in vivo* antitumor efficacy, the life span and the survival percentage of GC-51 injection was less than those of GC-51-loaded submicron emulsion at the same administration dosage, which also indicates that submicron emulsion might be a potential vector for GC-51 with enhanced *in vivo* antitumor effect.

4. Conclusion

In conclusion, a novel lead compound-based submicron emulsion was designed to act as the drug carrier for Cheliensisin A (GC-51) in the current study, which can be effectively delivered to solid tumors via parental administration route. The lyophilized negatively charged submicron emulsion droplets developed in this study were shown to produce certain therapeutic effects on colon metastized lung cancer models in BALB/c mice. In short, GC-51 is a potent antitumor drug and with its novel chemical structure, it may become a new lead compound, but further studies still need to be conducted to explore its *in vivo* metabolic profiles. Perhaps, certain structure modifications and preparation optimization are needed to further improve its *in vivo* therapeutic efficacy.

In addition, this study opens a new prospective for the delivery of hydrophobic and instable drugs. As a drug carrier, lyophilized submicron emulsion has efficiently overcome the *in vitro* instability of GC-51 and sustained its *in vitro* drug release, which improved both the *in vitro* and *in vivo* antitumor efficacy of GC-51. Submicron emulsion-based targeted drug delivery system may have wide applications, and therefore, represents a novel versatile tool for cancer treatment.

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