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RESEARCH PAPER

Evaluation of Docetaxel-Loaded Intravenous Lipid Emulsion: Pharmacokinetics, Tissue Distribution, Antitumor Activity, Safety and Toxicity

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ABSTRACT

Purpose The purpose of this study was to carry out a detailed evaluation of an intravenous lipid emulsion for docetaxel (DLE) without Tween 80 before clinical administration.

Methods The pharmacokinetics in rats and beagle dogs, tissue distribution, antitumor activity, safety test and toxicity of DLE have been investigated systematically to evaluate the formulation and compared with Taxotere® (DS).

Results The pharmacokinetic study in rats revealed that DLE exhibited higher plasma concentrations and AUC than DS, and a good correlation was observed between AUC and dose, while, in beagle dogs, the DLE was bioequivalent to DS. The tissue distribution study showed that the profiles of the two formulations were similar, indicating the DLE did not change the distribution of docetaxel *in vivo*. Furthermore, DLE was as safe as DS in the safety investigation and displayed significant antitumor activities against the A549, BEL7402 and BCAP-37 cell lines in nude mice, similar to DS. The corresponding results of the long-term toxic study demonstrated the DLE was less toxic than DS, and the toxic effects could be reversed.

Conclusions The DLE investigated in this paper was found to be an attractive new formulation and an appropriate choice for the clinical administration of docetaxel.

KEY WORDS docetaxel · evaluation · lipid emulsion

ABBREVIATIONS

A549	the A549 human pulmonary adenocarcinoma cell line
ALB	albumin
ALP	alkaline phosphatase
ALT	alanine transaminase
AST	aspartate transaminase
BCAP-37	the BCAP-37 human breast cancer cell line
BEL7402	the BEL7402 human hepatocellular carcinoma cell line
BUN	blood urea nitrogen
CK	creatinine kinase
Cr	creatinine
CT	coagulation time
DC	WBC differential count
DLE	lipid emulsion for docetaxel
DS	Taxotere
DTX	docetaxel
GCT	gama glutamyl transferase
GLB	globulin
GLU	glucose
HB	hemoglobin
HCT	hematocrit
MCHC	mean corpuscular hemoglobin concentration
MCV	mean corpuscular volume
PLT	blood platelet count
RBC	the red blood cell count
Ret	reticulocyte count
RTV	the relative tumor volume
T/C	the percentage of tumor growth rate
TBIL	total bilirubin
TBME	tert-butyl methyl ether
TCHO	total cholesterol
TG	triglyceride
TGI	the percentage of tumor growth inhibition rate
TP	total protein
U-BIL	bilirubin in urine
URO	urobilinogen in urine
WBC	white blood cell count

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INTRODUCTION

Docetaxel (DTX), an analogue of paclitaxel, was first semi-synthesized from 10-deacetyl baccatinIII, extracted from the needles of the European yew tree (*Taxus baccata* L.) in 1986 (1). It is a taxoid cytotoxic agent that promotes tubulin assembly into microtubules, stabilizes microtubules and inhibits their depolymerization, thereby causing mitotic arrest in the G_2/M phase of the cell cycle and, subsequently, cell death (1). Today, the drug, given by intravenous or intraperitoneal injection, has contributed significantly to the treatment of a variety of malignancies, such as ovarian, breast, gastric, and non-small-cell lung cancers (NSCLC), as well as head and neck cancer and some other cancers (2).

Because DTX is highly lipophilic and practically insoluble in water, at present, the main marketable products (Taxotere) used clinically are formulated in polysorbate 80 (Tween 80), requiring dilution prior to use with 13% ethanol in water; then it can be administered after further dilution in 5% dextrose solution or normal saline to a defined concentration of 0.3–0.74 mg/mL. It is said in the instructions for Taxotere that there may be foam on top of the solution in the presence of Tween 80 or precipitation due to the high lipophilicity of DTX during the dilution, indicating that its clinical use is not completely safe. Normally, the solution must be used within 4 h because of its instability. This is a great limitation of Taxotere for clinical application. In addition, Tween 80 is known to alter membrane fluidity, resulting in an increase of membrane permeability, which is associated with serious hypersensitivity reactions and cumulative-fluid retention (3). Apart from this, Tween 80 is able to interfere with P-glycoprotein, causing interaction with some associated drugs, such as epirubicin (4). Taking these findings into consideration, it is necessary to develop a novel and safe formulation for intravenous administration of DTX which does not involve Tween 80 and organic solvents for clinical treatment.

Recently, alternative approaches have been investigated to load DTX into liposomes (5), cyclodextrins (6), mixed micelles (7), submicron emulsion (8) and nanoparticles (9). However, most of these approaches have been clinically unsuccessful because of the low entrapment efficiency, complicated preparation procedure and poor physicochemical stability during long-term storage. In particular, these new dose forms mentioned above are not able to withstand thermal sterilization, which is replaced by sterile filtration using a 0.22 um Nalgene® nylon filter. This considerably restricts their industrial-scale production and clinical applications.

In the past few decades, the lipid emulsions formed mainly by soya bean oil and phospholipids have been found to be safe and excellent drug carriers for antitumor agents, owing to their high drug loading capacity, suitability for industrial-scale production and stability during long-term storage (10,11). Furthermore, intravenous lipid emulsions avoid direct contact of drugs with body fluids and tissues by loading the drugs in the internal phase (12), showing versatile advantages over conventional dose forms, such as reduced irritation or toxicity of the incorporated drugs, the possibility of sustained release, a reduction in drug hydrolysis and no precipitation during administration (13–15). Therefore, the use of lipid emulsions as a carrier is expected to be suitable for i.v. administration of DTX. Loading DTX in lipid emulsions could not only avoid the serious hypersensitivity reactions caused by Tween 80, but also be stable, safe and convenient for clinical administration. Therefore, the development of a DTX-loaded intravenous lipid emulsion should be a worthwhile and promising strategy. After detailed investigations in our laboratory, the DTX-loaded intravenous lipid emulsion, without Tween 80, produced by high-pressure homogenization was shown to be stable following autoclaving at 121°C for 10 min and remained stable during a 12-month storage period at 6±2°C.

The objective of the present study is to evaluate the intravenous lipid emulsion with a high drug entrapment efficiency involving DTX (DLE) mentioned above in terms of its pharmacokinetics, tissue distribution, antitumor activity, safety and long-term toxicity, in comparison with DS. All the results obtained strongly supported the feasibility of using DLE for clinical applications owing to its biological equivalence with the commercially available product DS and showed that it was similar to DS with regard to tissue distribution, antitumor activities and safety, but less toxic than DS.

MATERIALS AND METHODS

Materials and Animals

The following materials were purchased from the sources in parentheses: Docetaxel and Paclitaxel (Shanghai sanwei Pharma Ltd. Co., Shanghai, China), MCT and Oleic acid (Lipoid KG, Ludwigshafen, Germany), Egg lecithin (EPI-KURON 170, PC72%, Degussa Food Ingredients, Germany), Long-chain triglyceride (LCT) (Tieling Beiya Pharmaceutical Co., Tieling, China), Poloxamer 188 (Pluronic F68®) (BASF AG, Ludwigshafen, Germany), Glycerol (Zhejiang Suichang Glycerol Plant, Zhejiang, China), Sodium bisulfite (Tianjin Boya Chemical Industry Ltd. Co., Tianjin, China), Taxotere (Docetaxel for Injection) (Jiangsu Hengrui Medicine Ltd. Co., Lianyungang, China), tert-butyl methyl ether (TBME, Sinopharm Chemical Reagent Ltd. Co., Shenyang, China), formic acid (Dima Technology Inc., Richmond Hill, USA), methanol, acetonitrile and dehydrated alcohol (Tianjin Concord Technology Ltd., Co., Tianjin, China), egg albumin (Sigma-Aldrich Co., USA). All chemicals and reagents used were of analytical or chromatographic grade.

The pharmacokinetic evaluation of DLE was performed on rats and beagle dogs, while the tissue distribution experiment was carried out in mice. BALB/c-nu nude mice were used to study the antitumor activities of DLE, and rabbits, rats and guinea pigs were applied for the irritation assessment, hemolysis testing and hypersensitivity reaction, respectively, while rats were selected for the long-term toxicity study. The rats, mice, rabbits and guinea pigs used were purchased from the Experimental Animal Center (Shenyang Pharmaceutical University, Shenyang, China), the beagle dogs were provided by the Research Institute for Laboratory Animal in Kangping (Shenyang, China), while BALB/c-nu nude mice were provided by the Experimental Animal Center of Medical Department of Beijing University (Beijing, China). The A549 human pulmonary adenocarcinoma cell line and the BEL7402 hepatocellular carcinoma cell line were obtained from the Department of Pharmacology of the Cancer Institute (Chinese Academy of Medical Sciences, Beijing, China). The BCAP-37 human breast cancer cell line was supplied by the First Department of Pharmacology in the Institute of Materia Medica (Chinese Academy of Medical Sciences, Beijing, China). All the animal experiments mentioned above were evaluated and approved by the University Ethics Committee for the use of laboratory animals and in compliance with the Guidelines for the Care and Use of Laboratory Animals.

Preparation and Characterization of DLE

The preparation of the lipid emulsion containing DTX was as follows: First, the oil phase, which was composed of 2.5% (w/v) soybean oil, 7.5% (w/v) MCT, 3.0% (w/v) egg lecithin, 0.025%(w/v) oleic acid and 0.08% (w/v) DTX, was heated at 75°C with stirring until a uniform system was obtained. At the same time, 0.2% (w/v) F68, 0.02% (w/v) sodium bisulfite and 2.5% (w/v) glycerin were dispersed in water with stirring at 75°C to form the aqueous phase. Subsequently, the coarse emulsion was prepared by adding the oil phase to the aqueous phase with high-speed shear mixing (ULTRA RURRAX®IKA® T18 basic, Germany) at 15,000 rpm for 5 min. Then, the pH was adjusted to 5.5, and the volume was made up to 100% with double-distilled water. After that, the coarse emulsion was subjected to high-pressure homogenization (Niro Soavi NS10012k homogenization, Via M. da Erba, 29/A-43100 PARMA, Italy) at 800 bar for 10 cycles to obtain a fine emulsion. Finally, the emulsion was sealed in vials after flushing with nitrogen gas and autoclaved at 121°C for 10 min.

The DTX lipid emulsions showed a uniform milky appearance. The mean particle size of the DLE was 144.4± 41.90 nm, exhibited by the In-Wt Gaussian distribution. The zeta potential of DLE was −29.36 mV, and the pH value was 5.34. Also, the chemical stability was evaluated by the drug entrapment efficiency and the content of DTX in DLE, with results of 96.9% and 100.7% (the standard specification is 0.8 mg/mL), respectively.

Pharmacokinetic Study in Rats

A pharmacokinetic study in rats was designed to evaluate the DLE by comparison with the commercial product Taxotere (DS). Wistar rats weighting 180–220 g were divided into 6 groups with 6 rats (3 male and 3 female) in each group. Because the standard clinical dose of Taxotere for human is 75 mg/m^2, the dose for rats turns out to be 10 mg/kg, transferred from the skin surface area conversion. The DLE was administered at a concentration of 0.8 mg/mL, while the DS was first diluted by an initial dilution with 13% ethanol in water, and then further diluted to 0.8 mg/mL with normal saline. Taking 10 mg/kg as the middle dose, the first three groups were given DLE at an intravenous bolus dose of 5 mg/kg, 10 mg/kg and 20 mg/kg via the femoral vein, while the remaining three groups were administered DS at the same dose as the DLE groups. For the 20 mg/kg group, the intravenous bolus was allowed to run for 2 min, while the duration time of injection was 1 min for the 5 mg/kg and 10 mg/kg groups. At pre-determined time points (5 min, 15 min, 30 min, 1 h, 1.5 h, 2 h, 4 h, 6 h, 8 h and 12 h), blood samples of about 0.5 mL were collected into heparinized Eppendorf tubes by retro-orbital puncture, and then immediately centrifuged at 4000 rpm for 15 min to obtain plasma samples. The plasma samples were stored at −80°C until analysis. Aliquots (100 μL) of plasma were used for extraction and drug determination by UPLC/MS/MS.

Pharmacokinetic Study in Beagle Dogs

The pharmacokinetic study in beagle dogs was based on a single-dose, randomized, two-period crossover design carried out at intervals of 3 weeks. Six healthy beagle dogs weighing 8–12 kg, with equal numbers of males and females, were used in this study and were divided into two groups according gender (3 dogs in each group). The beagle dogs were fasted but allowed free access to water overnight before drug administration. The two groups of dogs were randomly selected to receive DLE or DS at an intravenous infusion dose of 3.4 mg/kg for 1 hour, which was also calculated from the skin surface area conversion at the dose of 75 mg/m^2 for clinical administration of 3-weekly regimen. The DLE and DS administrated to the beagle dogs were the same as that given to the rats in Pharmacokinetic Study in Rats. The intravenous drip was allowed to run for 1 h, and blood samples of about 2 mL were collected from the foreleg vein into heparinized centrifuge tubes, at 5 min, 15 min, 30 min, 45 min and

60 min during the intravenous drip period (mid infusion), and 5 min, 15 min, 30 min, 45 min, 1 h, 2 h, 3 h, 4 h, 6 h, 8 h and 12 h after the intravenous drip (post infusion). Subsequently, plasma samples were obtained following centrifugation at 4,000 rpm for 15 min and then stored at −80°C until determination. Aliquots (200 μL) of plasma were used for extraction and analysis by UPLC/MS/MS.

Tissue Distribution Study in Mice

The tissue distribution study was carried out on mice weighing 18–22 g, which were divided into 12 groups with 6 mice in each group, (equal numbers of males and females). DLE was given to the first six groups (DLE groups) at a dose of 16 mg/kg via the tail vein, which is transferred from the clinically used dose of 75 mg/m^2, while DS was given to the other six groups (DS groups) intravenously at the same dose level as the DLE groups. After administration, blood samples were collected by retro-orbital puncture from six mice each in the DLE and DS groups; thereafter, the mice were immediately sacrificed, and organs (heart, liver, spleen, lung, kidney, brain, stomach and intestine) were removed at 5 min, 30 min, 1 h, 4 h, 8 h and 12 h. The blood samples were centrifuged at 4,000 rpm for 15 min to separate the plasma, and the tissue samples were rinsed in ice-cold normal saline, blotted dry with filter paper, and then stored at −80°C along with the plasma until analysis.

Plasma and Tissue Sample Analysis

Sample Preparation

To determine the DTX concentration, 20 μL PTX methanol solution (20 ug/mL) was used as an internal standard and added to 100 μL rat plasma, 200 μL beagle dog plasma or 200 μL mouse tissue homogenate. After vortex-mixing for 1 min with a Liquid Fast Mixer (YKH-3, Jiangxi Medical Apparatus and Instrument Factory, China), 3 mL tert-butyl methyl ether (TBME) was added, and then extraction was performed by vortexing for 10 min. Following centrifugation at 4000 rpm for 10 min, the supernatant was transferred to a clean centrifuge tube and evaporated to dryness in a centrifugal concentrator under reduced pressure at 40°C (Labconco Corp., Missouri, USA). The residue was reconstituted in methanol, and a 5 uL aliquot was injected into the UPLC/MS/MS for analysis. The tissue homogenate mentioned above was prepared as follows: around 0.1 g of the tissue samples were accurately weighed, and 0.5 mL normal saline was added, then homogenized by a homogenizer (DY89-II, Xinzhi biological and scientific company, Ningbo, China) to obtain the final tissue homogenate.

Sample Determination by UPLC -MS/MS (16)

Chromatography was performed on an ACQUITY^{TM} UPLC system (Waters Corp., Milford, MA, USA) with a conditioned autosampler at 4°C. The separation of the DTX and PTX was achieved by using an ACQUITY^{TM} UPLC BEH C18 column (50×2.1 mm i.d., 1.7 μm; Waters Corp., Milford, MA, USA) at a column temperature of 35° C. The determination was carried out by gradient elution using acetonitrile (A) and water (B, containing 0.1% formic acid) as the mobile phase at a flow rate of 0.2 mL/min, and the total run time was 2.5 min. The gradient conditions are shown in Table I. The injection volume was 5 μL, and the partial loop mode was selected for sample injection.

The Waters ACQUITY^{TM} TQD triple-quadrupole tandem mass spectrometer (Waters Corp., Manchester, UK) was connected to the UPLC system via an electrospray ionization (ESI) interface. The samples were analyzed using an electrospary probe in the positive ionization mode with the capillary voltage set at 2.5 kV. The extractor and RF voltages were 3.0 and 0.1 V, respectively. The temperature of the source and desolvation was set at 100 and 400°C, separately. High-purity nitrogen was used as the desolvation gas (500 Lh^{-1}) and cone gas (50 Lh^{-1}). For collision-induced dissociation (CID), argon was used as the collision gas at a flow rate of 0.25 mL/min. Multiple reaction monitoring (MRM) mode was used for quantification. Transition reactions were 808.25→527.17 for DTX and 854.54→285.96 for the internal standard PTX. All data collected in centroid mode were acquired using Masslynx^{TM} NT4.1 software (Waters Corp., Milford, MA, USA). Post-acquisition quantitative analyses were performed using a QuanLynx^{TM} program (Waters Corp., Milford, MA, USA).

The method was validated for the determination of DTX in plasma and tissue samples, using the biological matrix. The precision and accuracy of the method at each concentration was calculated as the percentage relative standard deviation (%RSD). The lowest standard on the calibration curve with a concentration of 5 ng/mL was

Table I Gradient Conditions for UPLC

Time (min)	Flow rate (mL/min)	A (%)[a]	B (%)[b]	Curve
Initial	0.2	50	50	Initial
0.9	0.2	20	80	6[c]
1.6	0.2	20	80	6
1.7	0.2	50	50	6
2.5	0.2	50	50	6

[a] acetonitrile

[b] 0.1% formic acid solution

[c] linear

indentified as the lower limit of quantification (LLOQ) when the analyte peak was identifiable and reproducible with a precision of 7.5%. The calibration curve for DTX was linear over the range 5–50,000 ng/mL, while the intra-day precision at three concentrations (10, 1,000, 40,000 ng/mL) was 1.8–11.6%, and the inter-day precision was less than 15%, showing acceptable precision and accuracy. The relative recoveries from the plasma and tissue samples were determined by comparing the concentration of extracted samples at three concentrations (10, 1000, 40,000 ng/mL) with unextracted standards containing the same amount of DTX, giving a result of 67.9–78.2% and 71.2–83.4%, respectively.

Pharmacokinetics and Statistical Analysis

Pharmacokinetic analysis was carried out using drug and statistics (DAS) version 2.0 software (Mathematical Pharmacology Professional Committee of China, Shanghai, China). The statistical differences between two groups were evaluated by student's independent sample t-test and expressed as one-way p value. For all tests (including the studies described in the following paragraph), statistical significance was defined as p value < 0.05. The statistical analysis was performed using the statistical package for social science (SPSS, version 11.5). All descriptive parameters were expressed as mean ± SD.

Antitumor Activity *In Vivo*

In vivo antitumor efficacy of DLE was evaluated in nude mice bearing the A549 human pulmonary adenocarcinoma cell line, the BEL7402 human hepatocellular carcinoma cell line and the BCAP-37 human breast cancer cell line. Male nude mice were selected to estimate the activity against the A549 and BEL7402 lines, and female nude mice for the BCAP-37 line. Taking one of the three cell lines as an example, the experiments were designed as follows. One cell line was inoculated subcutaneously into the mice at the right axillary region, and the tumor was allowed to grow. When the tumor volume reached about 100 mm^3, the mice were randomly assigned to 5 groups based on the tumor size, with 7 mice in each group (day 0). Then, the treatment was started. The first three groups were given DLE at doses of 2 mg/kg (DLE-L), 4 mg/kg (DLE-M) and 8 mg/kg (DLE-H) via a tail vein twice a week for three weeks, so a total of 6 times, while the remaining two groups were given DS as a positive treatment group (DS) and DTX-free lipid emulsion as a negative control group (NC), respectively, at the same dose with the DLE-M group (4 mg/kg) in the same way. The body weight of the mice was monitored on day 7, 14 and 21 as an index of systemic toxicity. The tumor volumes were measured regularly twice a week (at day 4, 7, 11, 14, 18 and 23) using a Vernier's caliper (01010157, Zhongmeideli Co., Zhengjiang, China) across its two perpendicular diameters, and the volume calculated by the formula of $V = 1/2 \times L \times W^2$, where L is the length, W is the width. At the same time, the relative tumor volume (RTV) was calculated as $RTV = Vt/V_0$, where Vt indicated the mean tumor volume at day 4, 7, 11, 14, 18, 23, and V_0 was the mean tumor volume at day 0. Then, the percentage of tumor growth rate (T/C) was calculated by $T/C(\%) = T_{RTV}/C_{RTV} \times 100\%$, where T_{RTV} and C_{RTV} represented the RTV of the treated groups and the negative control group, respectively. At the end of the study (24 days after treatment was initiated), the mice were sacrificed by cervical dislocation, and tumors were collected and weighed to calculate the percentage of tumor growth inhibition rate (TGI), which was expressed as $TGI(\%) = (1 - Wt/Wc) \times 100\%$, where Wt and Wc denoted the mean final tumor weight of the treated groups and the negative control group(17). In summary, the antitumor activity was assessed by three evaluation criteria in terms of RTV, T/C and TGI. Moderate activity was defined as a $T/C\% \leqq 40\%$ and the TGI% value of 40% or more, as well as significant differences of RTV between the treatment groups and the negative control group. The antitumor activity study of each cell line was carried out twice to confirm the reproducibility and reliability.

Intravenous Injection Safety Test

Rabbit Ear Vein Irritation Assessment (15)

Twelve rabbits weighing 2.42±0.11 kg were divided into four groups with three in each group. The rabbits in the first two groups were administered DLE and DS injections with a daily dose of 0.64 mg/kg into the right ear marginal vein, respectively, which were repeated after 2 days. As a control, an equivalent volume of DTX-free lipid emulsion and normal saline were given in the same way to the other two groups. After injection, visual observations of the vascular reaction were recorded every day. The rabbits were killed 24 h after last dosing, and then the vascular tissues at the injection site were cut out and preserved in 10% buffer-formalin solution to prepare pathological sections for histopathological examination.

Hemolysis Test (18,19)

Hemolysis Test In Vitro. Rabbit blood was used to test the hemolysis effect of DLE *in vitro*. First, 100 mL of rabbit blood was collected from arteria cruralis, and fibrinogen was removed by shaking with glass beads. Then, 100 mL 0.9% normal saline was added to the fibrinogen-free blood sample, and the supernatant was removed after centrifugation at 1500 rpm for 15 min. The erythrocytes at the bottom of the

centrifuge tube were washed with normal saline three times as described above (centrifugation followed by redispersion). Finally, a 2% erythrocyte standard dispersion without fibrinogen was obtained by adding adequate amount of normal saline to the tube.

Different volumes of DLE and DS (0.8 mg/mL), 0.1, 0.2, 0.3, 0.4 and 0.5 mL, were added to the tubes pretreated with 2.5 mL 2% erythrocyte dispersion followed by 0.9% normal saline and made up to a final volume of 5 mL. As a comparison, 2.5 mL water as a positive control and 2.5 mL normal saline as negative control were added to 2.5 mL 2% erythrocyte dispersion. After vortex-mixing, the tubes were incubated at 37°C and then at 20 min, 40 min, 1 h, 2 h and 3 h, the tubes were centrifuged at 1,000 rpm for 10 min to obtain the supernatant, which was diluted and measured at 550 nm to obtain the optical density (OD)(752 UV-spectrophotometer, Beisida Instrument Factory, Chengdu, China). When the supernatant appeared to be red, it was concluded that a hemolysis reaction had occurred. However, a clear diaphanous supernatant indicated no hemolysis. In addition, the degree of hemolysis was compared with the OD value(20).

Hemolysis Test In Vivo. The hemolysis test *in vivo* was conducted in Wistar rats weighing 180–220 g, equal numbers of males and females, assigned into 3 groups (DLE group, DS group and DTX-free lipid emulsion group) with 8 rats in each group. The rats were given DLE and DS at a dose of 2.4 mg/kg, with DTX-free lipid emulsions of the same volume as a control, every three days for a total of 6 times. Then, 2 days after the last administration, the rats were sacrificed, and the blood and urine were collected to determine the red blood cell count (RBC), hematocrit (HCT), reticulocyte count (Ret), bilirubin in urine (U-BIL) and urobilinogen in urine (URO) (ACL-200, Blood auto-analyzer, BeckmanCoulter Corp., USA; Uritest-300, Uritest Corp, USA).

Hypersensitivity Reaction

Active Anaphylaxis (21). Thirty guinea pigs weighing 240~280 g were divided into five groups ($n=6$). The first two groups, as negative controls, were given normal saline solution and DTX-free lipid emulsion, respectively, while 0.3% egg albumin solution was given to the positive control group. DLE at doses of 1 mg/kg and 2 mg/kg, respectively, were injected into the other two groups. All the groups were intraperitoneally administrated every other day for three times. Ten days after the last injection, animals in each group were given a challenge dose of corresponding solution, which was 2-fold the administration dose, into the vein at the lateral of crus curvilineum. The animals were monitored for 3 h in order to record the hypersensitivity reaction after the challenge injection.

Passive Cutaneous Anaphylaxis (22). First, the antiserum was prepared as follows. Five groups (negative group, DTX-free lipid emulsion group, positive group, DLE-L and DLE-H groups) of rats weighing 180–220 g were given 0.5 mL DPT vaccine by intraperitoneal injection before the first sensitization. Then, a total volume of 1.0 mL 5% $Al(OH)_3$ in normal saline, DTX-free lipid emulsion and egg albumin solution of 2 mg/mL (prepared by adding appropriate egg albumin to 5% $Al(OH)_3$ in normal saline) were respectively given to the rats in the first 3 groups every 2 days with a total of 4 times, while 1.0 mL of DLE was injected into the rats in the DLE-L group, and 2.0 mL DLE was given to the rats in the DLE-H group in the same way. Then, 12 days after the last sensitization, blood was obtained from retro-orbital puncture of the rats, and the serum was pooled and stored at −80°C. Second, another 30 male rats were also assigned to five groups, the same as described above. The dorsal skin of each rat was clipped in four different places and 0.1 mL of 50-fold, 40-fold, 30-fold and 20-fold diluted antiserum was injected intracutaneously. At 48 h after sensitization, the positive group was given 1 mL of the challenge solution with 2 mg/mL egg albumin (prepared by adding appropriate egg albumin to 1% Evans blue solution) intravenously, while the negative group was given an injection of 1 mL 1% Evans blue solution, and the DLE and DTX-free lipid emulsion groups were injected 1 mL 2% Evans blue solution intravenously as the challenge solution. After 20 min, the rats were sacrificed, and the dorsal skin was collected to determine the diameter of the blue spot.

Long-Term Toxicity Study (23)

In order to provide references for clinical administration, the long-term toxicity study of DLE was carried out to investigate the toxic response, the target organ and the toxic reversibility, compared with DS. The rats were divided into 5 groups with 32 rats in each group (equal numbers of males and females): the DLE groups at high (DLE-H), middle (DLE-M) and low (DLE-L) doses, the DS group and the DTX-free lipid emulsion group. The three DLE groups were given DLE at doses of 28.8 mg/kg/3-week (DLE-H), 14.4 mg/kg/3-week (DLE-M) and 7.2 mg/kg/3-week (DLE-L), respectively, via intravenous injection every Monday and Thursday for 9 weeks, with every 3 weeks representing one regimen. In the same way, DS was given to another group at the same dose with the DLE-M group as the positive control group, while the group exposed to DTX-free lipid emulsion was taken as the negative control group (NC). After the second regimen (6 weeks) and the third regimen (9 weeks), 8 rats and 16 rats in each group, respectively, were sacrificed, and blood/

serum, urine and organ samples were collected for further assessment. Eight rats still alive in each group were subjected to a 2-week recovery test after the 9 weeks of treatment, and then the rats were killed after the recovery period. Throughout the whole experiment, the body weight and behaviors of all the rats were monitored and recorded. At the predetermined time points mentioned (after the second regimen, after the third regimen and after the recovery period), some of the blood samples obtained were anticoagulated for routine hematology examination, while the remaining blood samples were allowed to coagulate and then centrifuged to obtain serum for biochemical analysis, and, at the same time, urinalysis was also carried out. Furthermore, the sacrificed rats underwent systematic autopsies, and several organs were examined and weighed to obtain the organ coefficients (organ weight / body weight). Then, all organs and tissue samples were fixed in 10% formalin, embedded in paraffin, and 4 um sections were made, then stained with hematoxylin and eosin (HE) and observed under microscope in order to perform a histopathology examination.

In addition, the parameters in hematology examination were as follows: the red blood cell count (RBC), hemoglobin (HB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), reticulocyte count (Ret), white blood cell count (WBC), WBC differential count (DC), blood platelet count (PLT) and coagulation time (CT) (ACL-200, Blood autoanalyzer, BeckmanCoulter Corp., USA). The biochemistry analysis of the serum involved measurement of the aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), blood urea nitrogen (BUN), total protein (TP), albumin (ALB), globulin (GLB), creatinine(Cr), total bilirubin (TBIL), glucose (GLU), total cholesterol (TCHO), triglyceride (TG), creatinine kinase (CK), gama glutamyl transferase (GGT), K, Na and Cl (AU640, blood biochemical autoanalyzer, Olympus Corp., JAP). At the same time, urine was also tested with measurement of pH, nitrite, glucose, protein, urine-specific gravity, occult blood, bilirubin in urine, urobilinogen in urine, urinary ketone and white blood cell (Uritest-300, Urine analyzer, Uritest Corp, USA). In the autopsy study, the heart, lung, liver, spleen, kidney, brain, adrenal gland, thoracic gland, testes, epididymides, uterus and ovary were examined and weighed to calculate the organ coefficients, while the histopathology examination was conducted on the heart, lung, liver, spleen, kidney, brain (cerebellum, mesencephalon and brain stem), sciatic nerve, pituitary, spinal cord, adrenal gland, pancreas, stomach, duodenum, jejunum, ileum, colon, trachea, esophagus, thyroid gland, prostate gland, gonad (testes, epididymides, uterus and ovary), bladder, submandibular gland, sternum, thoracic gland, thoracic aorta, mesenteric lymph node and the tissue at the injection site.

RESULTS

Pharmacokinetic Study

Pharmacokinetic Study in Rats

The pharmacokinetic behavior of DLE was evaluated by determining the concentration of DTX in rat plasma after administration at an intravenous bolus dose of 5 mg/kg, 10 mg/kg and 20 mg/kg, while Taxotere (DS) was chosen as a comparison. Convulsion was observed in four male rats of the DS group immediately after administration, but disappeared within 5 min. Analysis of the data obtained was first carried out using a compartmental model, and the results fitted a three-compartment model with a weight factor of 1/cc. Then, the pharmacokinetic parameters were calculated by the statistical moment method. The mean plasma concentration-time profiles of DLE and DS at different doses obtained from measurements are shown in Fig. 1, and the main pharmacokinetic parameters are summarized in Table II. These results are consistent with the literature other scientists reported (8,24). As seen from the figures, the DTX concentration in plasma rapidly decreased in DLE group, but it was significantly higher than that of the DS group during the first hour. Comparison of the pharmacokinetic parameters showed that the DLE group had markedly higher AUC, smaller clearance (CL) and lower apparent volume of distribution (Vss) than the DS group ($p<0.05$). However, half-lives ($t_{1/2}$) of DLE and DS were similar at the three dose levels.

In addition, a dose correlation study suggested that the AUC was dose proportional within the experimental doses, although there is a trend towards reduced clearance at the highest concentration (20 mg/kg). The reduced clearance is in accordance with the nonlinear elimination of DTX, which has been reported by the isolated perfused rat liver at a concentration of 5–50 μmol/L (25–27).

Pharmacokinetic Study in Beagle Dogs

Based on the determination of DTX in dog plasma, it was concluded that the mean plasma concentration-time profiles of DLE and DS depicted in Fig. 1 also fitted a three-compartment model, similar to the rats. The main pharmacokinetic parameters calculated by the non-compartment model are presented in Table III. As shown in Fig.1 and Table III, the parameters were comparable for both DLE and DS. The AUC of DLE was slightly higher than that of DS; nevertheless, the difference between them was not significant ($p>0.05$). The CL and Vss of DLE were 2.02 and 1.49 times lower than that of DS, respectively, while there was no dramatic difference in the half-life of these two different formulations. The CL obtained from this study

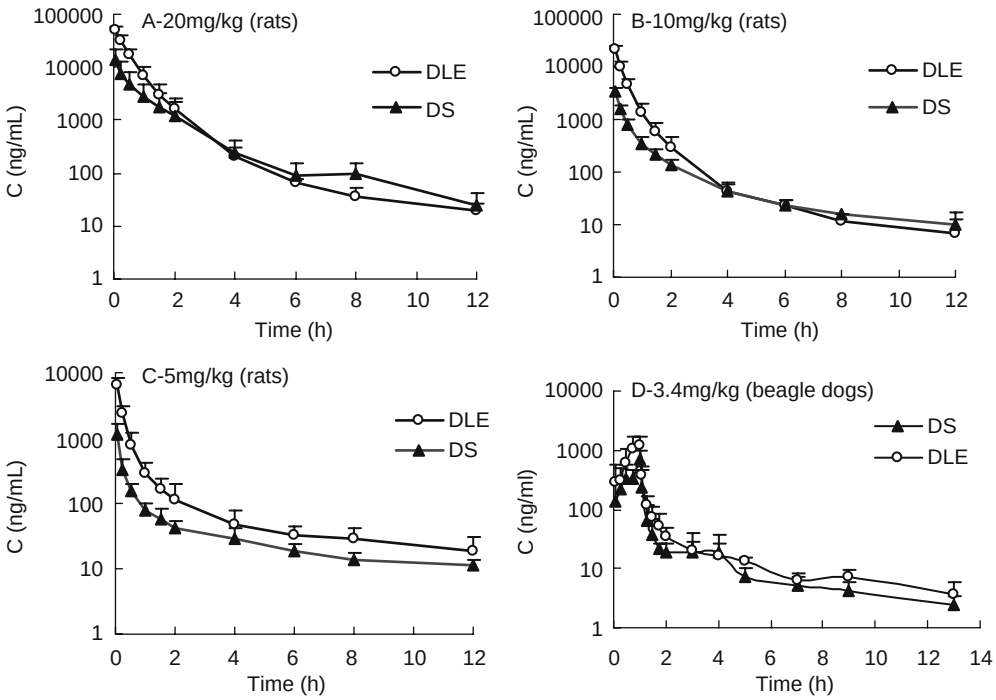

Fig. 1 Mean plasma concentration-time profile of DTX after i.v. administration of DLE and DS at doses of 20 mg/kg, 10 mg/kg and 5 mg/kg to rats and 3.4 mg/kg to beagle dogs ($n = 6$).

displayed high individual variability over a range of values from 1.882 to 10.452 L/h/kg, which agreed with previous findings (1,28). Furthermore, according to the FDA guidance, the DLE was bioequivalent to the Taxotere (DS) in beagle dogs, with the AUC and Cmax ratio's 90% confidence interval ranged from 0.80 to 1.25.

In addition, during the infusion process, all the six dogs in the DS group exhibited acute hypersensitivity reactions including swollen snout, facial erythema, dysphoria accompanied with scratching and shaking head behavior, and also discontinuity light stool, which disappeared after drip, while the DLE group did not show any of these symptoms. These acute hypersensitivity reactions in the DS group may be attributed to the injection vehicle (Tween 80) (29); hence, DLE, a novel and safe drug carrier, was more appropriate for DTX than DS. After one period of administration of DLE and DS, toxic effects involving emesia, diarrhea and anorexia were observed in all six dogs. Glucose injections of 500 mL one day were given to supply

Table II Pharmacokinetic Parameters After I.v. Administration of DLE and DS at Doses of 20 mg/kg, 10 mg/kg and 5 mg/kg to Rats ($n = 6$)

	Unit	DLE	DS
Dose	(mg/kg)	5	5
AUC[a]	(μg /L h^{-1})	2545.3 ± 887.3	593.0 ± 216.4
AUMC[a]	(μg/L)·h^2	2175.5 ± 1166.7	902.1 ± 413.3
$t_{1/2}$	(h)	3.0 ± 1.2	2.7 ± 0.7
CL[a]	(L/h/kg)	2.1 ± 0.6	8.8 ± 3.1
Vss[a]	(L/kg)	9.0 ± 4.6	33.7 ± 15.0
Dose	(mg/kg)	10	10
AUC[a]	(μg /L h^{-1})	9267.4 ± 1836.7	1887.4 ± 294.9
AUMC[a]	(μg/L)·h^2	4461.4 ± 1519.7	2131.3 ± 602.7
$t_{1/2}$	(h)	2.3 ± 1.5	2.2 ± 0.7
CL[a]	(L/h/kg)	1.1 ± 0.2	5.3 ± 0.8
Vss[a]	(L/kg)	3.4 ± 1.6	17.4 ± 7.0
Dose	(mg/kg)	20	20
AUC[a]	(μg /L h^{-1})	29496.1 ± 7517.2	10405.7 ± 7321.4
AUMC[a]	(μg/L)·h^2	18705.7 ± 7279.0	12212.6 ± 9671.4
$t_{1/2}$	(h)	2.2 ± 0.9	1.9 ± 1.1
CL[a]	(L/h/kg)	0.7 ± 0.2	3.2 ± 2.2
Vss[a]	(L/kg)	2.3 ± 1.1	7.0 ± 3.7

AUC area under the concentration-time curve; *AUMC* area under the cross-product of the time and plasma concentration-time curve; $t_{1/2}$ half time; *CL* clearance; *Vss* apparent volume of distribution at steady state.

[a] $p < 0.05$

Table III Pharmacokinetic Parameters After I.v. Drip (1 h duration) Administration of DLE and DS at a Dose of 3.4 mg/kg to Beagle Dogs ($n = 6$)

	Unit	DLE	DS
Dose	(mg/kg)	3.4	3.4
AUC	(μg /L h^{-1})	927.3 ± 448.3	517.1 ± 266.6
AUMC	(μg/L)·h^2	1211.1 ± 495.1	532.6 ± 275.5
$t_{1/2}$	(h)	5.9 ± 3.1	4.2 ± 1.1
CL[a]	(L/h/kg)	4.1 ± 1.6	7.4 ± 2.5
Vss	(L/kg)	31.5 ± 12.8	42.2 ± 11.8

AUC area under the concentration-time curve; *AUMC* area under the cross-product of the time and plasma concentration-time curve; $t_{1/2}$ half time; *CL* clearance; *Vss* apparent volume of distribution at steady state.

[a] $p < 0.05$

energy and nutrients to these dogs, and they all gradually recovered after 13 days.

Tissue Distribution Study

The tissue DTX concentrations *versus* time after i.v. administration of DLE and DS at a dose of 16 mg/kg to mice are presented in Fig. 2A–I. The concentrations of DTX in different tissues at 0.083, 0.5, 1, 4, 8 and 12 h after administration of DLE and DS are shown in Fig. 3, while the AUC values in various tissues of the two formulations are listed in Table IV. At 5 min after administration, the highest level of DTX was observed in all collected tissues, with the highest concentrations being found in the lung, followed by liver, kidney, spleen, heart, intestine, stomach and brain (Fig. 3). In addition, it can be seen from the Fig. 2i that the mean plasma concentration-time profiles of DLE and DS in mice were similar to those in rats (see Pharmacokinetic Study in Rats), both indicating that DLE produced higher plasma concentration than DS at initial time points. The similar distribution patterns for both formulations demonstrated that DLE did not alter the tissue distribution behavior of DTX in mice significantly in comparison with DS. When DLE and DS were given to mice, the drug concentration in lung was the highest at the initial time point of 5 min for both formulations. In liver, the amount of DTX was second highest following that in lung at the 5 min time point, then it fell quickly at 0.5 h due

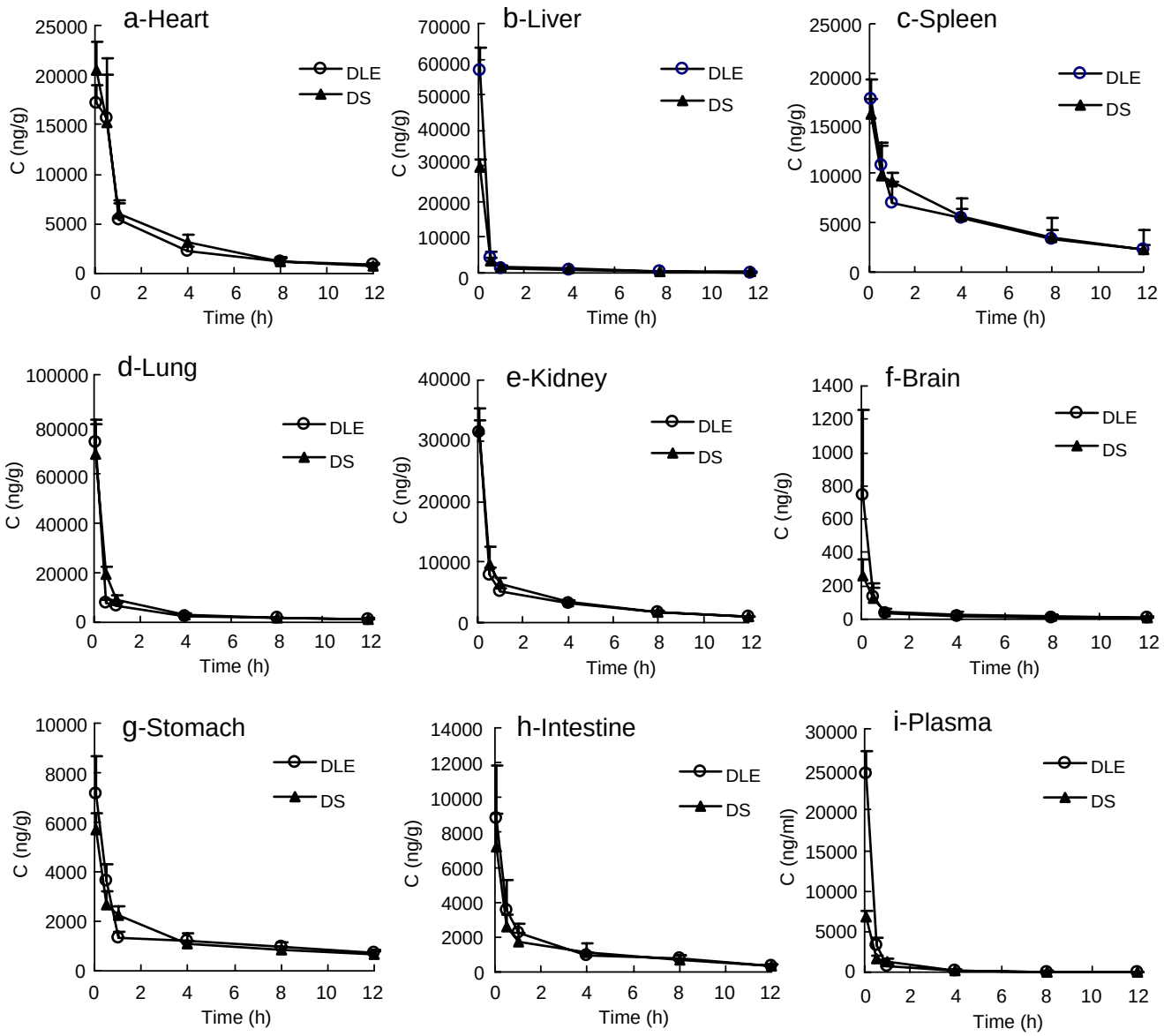

Fig. 2 Tissue distribution curves of DTX after i.v. administration of DLE and DS of 16 mg/kg to mice ($n = 6$).

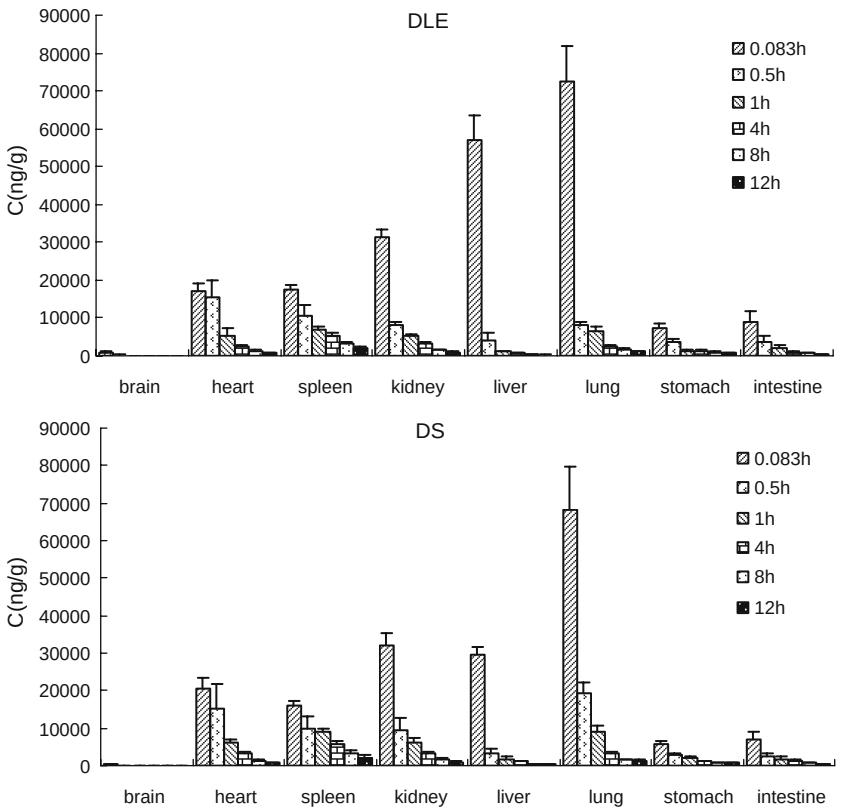

Fig. 3 Concentrations of DTX in different tissues at 0.083, 0.5, 1, 4, 8 and 12 h after i.v. administration of DLE and DS of 16 mg/kg to mice ($n = 6$).

to the rapid metabolism of DTX by liver microsomes implicate cytochromeP450 enzyme systems (CYP3A4) (29). The dramatic decrease in DTX resulted in a shorter residence time for DTX in liver, causing the relative low AUC of both DLE and DS. Moreover, there was a higher DTX concentration in liver for DLE than DS, which further confirmed the uptake of emulsion droplets by the RES in liver. Although there were quantitative differences, the tissue distribution profiles were similar for the two formulations.

Table IV AUC Values in Various Tissues After I.v. Administration of DLE and DS at a Dose of 16 mg/kg to Mice ($n = 6$)

Tissues	AUC(mg/g h^{-1})	
	DLE	DS
Heart	36.7 ± 3.6	41.0 ± 5.4
Liver[a]	25.1 ± 2.7	19.2 ± 2.6
Spleen	59.2 ± 5.5	62.7 ± 5.6
Lung[a]	53.0 ± 4.4	64.6 ± 4.9
Kidney	41.5 ± 1.9	45.4 ± 3.1
Brain	0.5 ± 0.2	0.4 ± 0.1
Stomach	15.7 ± 1.7	15.3 ± 0.5
Intestine	15.2 ± 1.8	13.5 ± 1.0

[a] $p < 0.05$

Antitumor Activity *In Vivo*

After the pharmacokinetics and tissue distribution evaluation, it can be expected that DLE may display analogous effects to DS in clinical situations owing to their similar pharmacokinetic and distribution behaviors. In order to confirm that, the antitumor activities of DLE against A549, BEL7402 and BCAP-37 were investigated and compared with those of DS.

First, in the case of A549, the antitumor activities of DLE and DS were assessed in nude mice, and the values of RTV, T/C and TGI of all the tested groups are listed in Table V. It can be seen that, compared with the negative group, both the DLE and DS groups exhibited significant antitumor activities on A549, but there was no difference between the DLE and DS. In detail, during the study, piloerection and diminished vigor were observed in all the mice, and the body weight of both the treated groups and negative control group increased slowly, which may be caused by the toxicity of the transplanted tumor. As shown in Table V, the RTV of the negative control group was approximately 3–4 times the size of the treated groups at the end of the study, which clearly demonstrated that both DLE and DS were effective in inhibiting A549 tumor growth. These results also suggested that DLE and DS exhibited marked antitumor activity against A549 with a T/C less than 40% and a TGI higher than 40%, especially with a T/C lower than 20% in the DLE-H group and less than 30% in the DLE-M group. Moreover, the DLE-

Table V Three Evaluation Criteria of A549, BEL7402 and BCAP-37 in Different Groups at the End of the Study ($n=7$)

	NC	DS	DLE-H	DLE-M	DLE-L
cell line	A549	A549	A549	A549	A549
RTV [a]	23.6 ± 6.31	8.75 ± 5.08	3.75 ± 3.35	6.52 ± 6.67	7.89 ± 3.25
T/C (%)	–	37.1	15.9	27.6	33.4
TGI (%)	–	67.71	60.71	61.43	58.02
cell line	BEL7402	BEL7402	BEL7402	BEL7402	BEL7402
RTV [a]	20.24 ± 6.31	3.74 ± 3.93	2.84 ± 1.71	3.46 ± 1.17	5.02 ± 1.99
T/C (%)	–	18.46	14.1	17.08	24.81
TGI (%)	–	81.44	86.8	72.85	69.96
cell line	BCAP-37	BCAP-37	BCAP-37	BCAP-37	BCAP-37
RTV [a]	57.15 ± 17.18	18.42 ± 11.30	14.11 ± 4.98	18.52 ± 12.62	19.57 ± 7.93
T/C (%)	–	32.23	24.68	32.4	34.25
TGI (%)	–	51.9	57.04	52.19	40.45

RTV the relative tumor volume

T/C the percentage of tumor growth rate

TGI the percentage of tumor growth inhibition rate

[a] $p<0.01$ compared with the NC group

M and DS group at the same dose of 4 mg/kg exhibited a TGI value of 61.43% and 67.71%, respectively, confirming the moderate activity in this cell line without any significant difference ($p>0.05$). Subsequently, the antitumor activities against BEL7402 and BCAP-37 were found to be similar to that in A549, and the data are given in Table V. These results indicated that both DLE and DS exhibited significant antitumor activities to BEL7402 and BCAP-37, but there was no difference between the two groups.

Above all, it can be concluded that DLE displayed significant antitumor activities against A549, BEL7402 and BCAP-37, similar to DS. Among them, BEL7402 was found to be the most sensitive to DTX, followed by A549 and then BCAP-37. The similar antitumor activities of DLE and DS were consistent with the results of the tissue distribution study, in which both two formulations displayed analogous distribution behavior.

Intravenous Injection Safety Test

Rabbit Ear Vein Irritation Assessment

After a 2-day administration, there was no obvious visible damage such as erythema and edema at the injection site of all the groups. In addition, the histopathologic examination of the rabbit ear-rim vein showed that there was no angiectasia, thrombus or vascular congestion in blood vessel at or away from the site of injection. Furthermore, in the surrounding tissues there were no signs of hemorrhage, dropsy, inflammatory cell infiltrate or endothelial cell necrosis and degeneration. The phenomena mentioned above were similar in all the groups, indicating that no intravenous irritation was caused by both DLE and DS.

Hemolysis Test

Hemolysis Test In Vitro. During the 3 h observation, the tubes in the negative control group, DS groups and DLE groups appeared to be clear, with the erythrocyte precipitated at the bottom, while a red solution without erythrocyte at the bottom was observed in the tubes of the positive control group at 20 min, indicating complete hemolysis. In addition, the OD value of the supernatant did not show any significant difference after 3-hour incubation at 37°C at the different time points. These experimental phenomena demonstrated that both DLE and DS at different concentrations did not cause hemolysis or erythrocyte agglutination at 37°C.

Hemolysis Test In Vivo. There were no abnormal alterations in the values of RBC, HCT, U-BIL and URO obtained in both DLE and DS groups, compared with the DTX-free lipid emulsion group. However, the Ret value of the DLE and DS group fell markedly, mainly as the result of inhibition of DTX in the marrow hematopoietic cells. These results suggested that DLE and DS behaved similarly in the hemolysis test *in vivo*.

Hypersensitivity Reaction

In the active anaphylaxis study, three guinea pigs in the highest DLE dose group (2 mg/kg) died 2, 5 and 7 days after administration, respectively, due to the cytotoxicity of DTX. Following the challenge, all the guinea pigs in positive control group exhibited obvious hypersensitivity symptom including dyspnea and convulsion, and then died within 2 min. However, there was no anaphylaxis symptoms, such as nose scratching, sneezing, erect hair, twitching, dyspnea and convulsion in the negative control groups and the DLE group at a dose of 1 mg/kg. In the passive cutaneous anaphylaxis study, no blue spot appeared in the DLE groups. Therefore, it can be seen that the intravenous administration of DLE was safe and did not induce hypersensitivity reactions.

Long-Term Toxicity Study

From the results obtained above, it can be concluded that DLE is as safe as DS in terms of the irritation assessment, hemolysis test and hypersensitivity reaction, and it exhibited similar antitumor activities to DS, indicating that it is an attractive therapeutic option. However, before its further application in clinical trials, it is necessary to investigate the long-term toxicity and assess its toxic response, the target organ and the toxic reversibility.

After the 11-week long-term toxicity study, the results were described in three parts: 1) the general behavior and body weight, 2) hematology, serum biochemistry analysis and urinalysis, and 3) organ coefficients and histopathology examination.

General Behavior and Body Weight

During the whole treatment period, the rats in DLE-H, DLE-M, DLE-L and DS groups experienced reduced appetite, weight loss, emaciation and depilation. Although the abnormal behavior resolved to a certain extent during the recovery period, it failed to reach the level of the NC group. Briefly, the variations in body weight and general behavior of the rats in the DS group (14.4 mg/kg) were much more serious than those in the DLE-M group (14.4 mg/kg), and the data are shown in Table VI. In detail, taking the male rats as an example, the increase in the body weight (GR) in the DS group was 67.83 ± 20.41%, compared with 87.01 ± 14.16% in the DLE-M group after the second regimen, and this difference was clear. Likewise, after the third regimen there were also significant differences between the DS (103.03 ± 15.61%) and DLE-M (129.96 ± 13.94%) groups. In addition, the GR of DLE declined in a dose-dependent manner. During the recovery period, the rats displayed improved general behavior, increased appetite and body weight. However, the body weights of the rats in the DTX treatment groups remained lower than those in the NC group. The results suggest that both DLE and DS have side effects on the general behavior and body weight due to the toxicity of the DTX itself, whereas the effect of DS on body weights was much more severe than that of DLE.

Hematology, Serum Biochemistry Analysis and Urinalysis

The hematology examination results, summarized in Table VII, indicated that the values of RBC, HB, Ret and WBC in the DLE-M group showed fewer changes than the DS group and revealed a dose-dependent decrease in RBC, HB and WBC, but a increase in Ret. Based on the Table, it can be seen that, after the second regimen, the RBC, HB and WBC in the DS group decreased by 16.95%, 22.42% and 27.22%, respectively, compared with 5.04%, 3.06% and no obvious changes in the DLE-M group. However, the Ret in the DS group increased by 17.31 ± 8.09%, while the value was 6.62 ± 1.69% in the DLE-M group, compared with 3.11 ± 0.60% in the NC group. This strongly suggested that DLE was less toxic than DS. Furthermore, after the third regimen, the values of most of the serum biochemistry parameters are presented in Table VIII. From the table, the levels of TP, ALB and GLB in the DS, DLE-H and DLE-M groups decreased, while the TBIL and TCHO increased, compared with the NC group. In addition, the ALT, AST as well as the ALP levels, which remained unchanged in the DLE-M group, declined by 18.8%, 19.68% and 11.51%, respectively, in the DS group, while the other parameters were similar in all groups. During the recovery period, the abnormalities in both hematology and serum biochemistry restored to the level of the NC group, except the WBC, which recovered more slowly. There were no effects on urinalysis throughout the whole study. Therefore, it can be concluded that the effects of DS on RBC, WBC and TP were greater than those of DLE.

Table VI Increase in the Body Weight (%) of the Rats in All Groups at Different Time Points

	NC	DS	DLE-H	DLE-M	DLE-L
Time points	Male	Male	Male	Male	Male
6 weeks(2nd regimen)	180.26 ± 21.38	67.83 ± 20.41 [a]	31.56 ± 23.98 [a]	87.01 ± 14.16 [a, c]	118.08 ± 22.98 [a]
9 weeks(3rd regimen)	227.42 ± 29.72	103.03 ± 15.61 [a]	76.85 ± 13.82 [a]	129.96 ± 13.94 [a, c]	141.91 ± 20.40 [a]
11 weeks(recovery)	272.29 ± 33.91	133.80 ± 27.91 [a]	136.86 ± 34.71 [a]	153.43 ± 5.99 [a, c]	167.51 ± 22.15 [a]
	Female	Female	Female	Female	Female
6 weeks(2nd regimen)	83.60 ± 16.12	30.43 ± 14.98 [a]	23.75 ± 14.49 [a]	53.51 ± 17.52 [a, c]	65.99 ± 20.09 [b]
9 weeks(3rd regimen)	115.52 ± 15.83	38.79 ± 15.48 [a]	51.23 ± 11.88 [a]	65.73 ± 18.95 [a, c]	94.48 ± 21.42 [a]
11 weeks(recovery)	141.63 ± 26.21	72.60 ± 13.56 [a]	61.21 ± 19.91 [a]	80.90 ± 7.78 [a]	93.09 ± 26.23 [b]

[a] $p < 0.01$, [b] $p < 0.05$ compared with the NC group

[c] $p < 0.05$ between the DS and the DLE-M group

Table VII Hematology Parameters in All Groups After the Second Regimen

	NC	DS	DLE-H	DLE-M	DLE-L
RBC($\times 10^{12}$/L)	7.14 ± 2.00	5.93 ± 0.13	5.11 ± 1.39 [b]	6.78 ± 0.42 [c]	7.82 ± 0.35
HB(g/L)	155.00 ± 5.32	120.25 ± 5.33 [a]	108.25 ± 25.38 [b]	150.25 ± 13.23 [c]	148.75 ± 6.08
HCT (%)	49.53 ± 2.11	41.60 ± 1.87 [a]	36.68 ± 7.55 [a]	44.43 ± 1.22 [a, c]	46.23 ± 1.28
MCV(fL)	60.80 ± 1.47	70.18 ± 2.38 [a]	72.90 ± 7.37 [b]	65.65 ± 3.18 [b]	59.95 ± 1.65 [b]
MCHC(g/L)	313.25 ± 5.25	289.00 ± 3.74 [a]	293.75 ± 11.15 [b]	314.50 ± 6.35 [c]	318.00 ± 5.89
Ret (%)	3.11 ± 0.60	17.31 ± 8.09 [b]	15.01 ± 8.59 [b]	6.62 ± 1.69 [b, c]	3.79 ± 0.69
WBC($\times 10^{9}$/L)	10.43 ± 4.13	7.59 ± 2.58 [b]	5.51 ± 1.50 [b]	11.35 ± 1.31 [c]	11.38 ± 1.51
PLT($\times 10^{9}$/L)	725.35 ± 96.10	708.35 ± 77.70	404.25 ± 180.67 [b]	729.85 ± 83.18	705.10 ± 88.27
CT (s)	122.50 ± 24.08	184.00 ± 37.01 [b]	174.25 ± 22.99 [b]	134.25 ± 11.03 [c]	175.25 ± 47.95

[a] $p < 0.01$, [b] $p < 0.05$ compared with the NC group

[c] $p < 0.05$ between the DS and the DLE-M group

Organ Coefficients and Histopathology Examination

The organ coefficients and histopathology examination were carried out to study the organ damage induced by DLE or DS. After the third regimen, it can be seen that the organ coefficients of the thoracic gland, liver, lung, kidney, brain, tests and ovary in the DLE-H, DLE-M and DS groups were smaller than that in NC group, indicating atrophy in those organs, which was more serious in the DLE-H and DS groups. Furthermore, from the results of the histopathology examination, injury to hematopoietic cells in bone marrow was observed in the rats in the DLE-H and DS groups, and this was exhibited by a decrease in hematopoietic cells and an increase in adipocyte. However, there was no obvious injury to bone marrow in the DLE-M and DLE-L groups. In addition, the observations in terms of the atrophy in the cortex and the decline in lymphocytes appeared in splenic corpuscle, thoracic gland, lymph node and lymphocytes of rats in the DLE-H and DS groups. Besides, the gonads of rats in DLE-H and DS groups were slightly damaged, with the decrease of spermatid and spermatozoa in male rats, together with injury to follicle cells in female rats. Above all, it can be seen from the histopathology examination that the toxic target organs of both DLE and DS were the immune organs, including the thoracic gland, lymph nodes and spleen, hematopoietic organs involving bone marrow and the reproductive organs involving the testis and ovary. As for other tissues, there were no drug-related histopathological changes except for the lung, in which pulmonary inflammation was found in the DS group. During the recovery period, the atrophy of the organs gradually disappeared, the hematopoietic cells in bone marrow and lymphocytes markedly increased to normal levels and the damage to the gonads also recovered to some degree.

It can be concluded that the abnormal symptoms appearing in the DLE-M group mentioned above were less severe than those in the DS group, at the same dose as the DLE-M group, demonstrating a lower toxic effect than the market formulation DS. Furthermore, both the DLE and DS had the same target organs, including immune organs, hematopoietic organs and reproductive organs, which was in agreement with the results of the tissue distribution study.

Table VIII Main Serum Biochemistry Parameters in All Groups After the Third Regimen

	NC	DS	DLE-H	DLE-M	DLE-L
ALT(U/L)	76.86 ± 14.47	62.38 ± 14.99	65.08 ± 12.16	77.30 ± 15.41	71.99 ± 8.05
AST (U/L)	292.49 ± 42.17	219.15 ± 66.88 [b]	229.35 ± 40.08 [a]	285.16 ± 90.06	263.93 ± 46.55
TP (G/L)	68.76 ± 4.84	62.80 ± 3.56 [b]	62.25 ± 4.58 [b]	64.49 ± 2.45 [b]	69.63 ± 2.72
ALB(G/L)	37.75 ± 1.91	35.29 ± 1.88 [b]	35.35 ± 1.90 [b]	35.01 ± 0.90 [b]	35.39 ± 2.49
TBIL(Umol/L)	1.01 ± 0.36	1.12 ± 0.17	0.96 ± 0.29	1.02 ± 0.37	1.13 ± 0.26
ALP (U/L)	179.56 ± 42.56	158.90 ± 28.83	183.93 ± 57.27	195.24 ± 35.03 [c]	213.95 ± 39.03
GLB(g/L)	31.01 ± 3.06	26.94 ± 1.86 [a]	25.94 ± 3.10 [a]	29.09 ± 1.63 [c]	32.36 ± 1.74
TCHO(mmol/L)	1.49 ± 0.22	2.12 ± 0.26 [a]	2.14 ± 0.40 [a]	1.96 ± 0.35 [a]	1.83 ± 0.30 [b]

[a] $p < 0.01$, [b] $p < 0.05$ compared with the NC group

[c] $p < 0.05$ between the DS and the DLE-M group

DISCUSSION

In the pharmacokinetic study in rats, the concentration of DTX in the DLE group was much higher than that in the DS group during the first hour. From the pharmacokinetic parameters in Table II, the DLE group exhibited a 2.8–4.9-fold increase in AUC, a 4.2–4.7 times reduction in CL and a 3.0–5.2 multiple decrease in Vss. This was mainly because of the specific structure of lipid emulsions as a drug carrier. In case of the DLE, with loaded drug in the internal phase, it took time to be released from the emulsion droplets into blood. Hence, the DTX concentration determined in the DLE group involved the drug released and retained in the lipid emulsion, which was confirmed by the experimental phenomenon that the plasma obtained at the first points was white and milky. The results were consistent with the reports showing that the lipophilic drug when formulated in lipid emulsions showed higher plasma concentration after i.v. administration than solution forms (30). Therefore, the specific structure of DLE with DTX loaded in the internal layer resulted in differences in the AUC, CL and Vss between the two kinds of formulations to some extent. In short, the DLE, with incorporated DTX in the lipid core, successfully produced higher plasma concentration and AUC, so it may offer an improved therapeutic index compared with DS. Also the similar half-time at the three dose levels further indicated the linear pharmacokinetics of DTX within the experimental doses.

Following the evaluation of pharmacokinetic behavior of DLE in rats, the pharmacokinetic study in beagle dogs was also investigated at an intravenous drip dose of 3.4 mg/kg, compared with DS. From the results obtained, it can be seen that DLE and DS had similar pharmacokinetic behaviors in beagle dogs and in rats, which could also be interpreted as described above, except for the difference in the AUC of the two formulations. The difference in AUC in rats was significant, while it was not significant in beagle dogs. This may be related to the different methods of administration, with intravenous bolus injection in rats and intravenous drip injection in beagle dogs. In the case of rats, all the drugs were entirely given into blood vessel one time via an intravenous bolus injection. For the DLE, it took more time for drug to be released from the inter phase of the O/W structure into the blood, and there was a higher concentration at the initial time points, resulting in a significant difference between the DS and DLE in the rat AUC values, whereas in the case of beagle dogs, because DTX was gradually administered into blood vessels by intravenous drip injection both for DLE and DS, the drug concentration increased progressively instead of reaching a sudden peak. Although the DS group may show a relative low concentration of DTX than DLE initially, the drugs were still continuously infused into blood, easing up the fall in plasma concentration to some extent. Therefore, the concentration of DTX could be kept at a certain level during the drip procedure. Accordingly, there was no significant difference in AUC between DS and DLE in beagle dogs. In addition, in order to investigate the toxic reactions in beagle dogs described in Pharmacokinetic Study in Beagle Dogs, two beagle dogs, one in the DS group and the other in the DLE group, were dissected after the second period experiment, and then the heart, liver, spleen, lung, kidney, stomach, intestine and adrenal gland were collected to prepare the pathological sections. From the pathological section photos, it could be seen that there was mucosal necrosis in gastrointestinal tract of both dogs, while there were no abnormalities in other tissues. These results are consistent with the digestive tract lesions reported in other publications (29), indicating that the toxic effects observed in beagle dogs were mainly due to the drug itself, rather than the dosage form. Also, it was reported that the toxic dose low (TDL) in beagle dogs was 15 mg/m^2 (2), while the dose in this study was 75 mg/m^2; therefore, the toxic effects appearing in the experiment were acceptable and reasonable. The experimental phenomenon also indicated that the dogs were more sensitive to DTX than rats (31).

Further, a comparative tissue distribution investigation was carried out between DLE and DS by measuring the concentration of DTX in various mouse tissues, including heart, liver, spleen, lung, kidney, brain, stomach and small intestine. When DLE and DS were given to mice, the drug concentration in lung was the highest at the initial time point in comparison with all other tissues investigated. Also from Fig. 3 it can be seen that DTX has relatively high drug disposition in lung, supporting its good therapeutic effect for the treatment of non-small-cell lung cancer. Moreover, the passive targeting of DLE to liver might suggest an enhanced therapeutic effect on liver cancers, which still needs further investigation. Above all, DLE was a novel and safe drug carrier which did not alter the distribution of DTX *in vivo*. Furthermore, the analogous distribution of DLE and DS resulted in similar antitumor activities against human A549, BEL7402 and BCAP-37 cell lines (Antitumor Activity *In Vivo*). In the safety test, it can be concluded that DLE, which exhibited no irritation, hemolysis or hypersensitivity, was as safe as the commercial products DS.

In the long-term toxicity study, the damage caused by DLE in terms of hematology and serum biochemistry was less serious than that of DS. This may be attributed to the specific structure of the lipid emulsion, with drugs loaded in the internal phase, preventing the direct contact of drugs with the blood and body fluids. On the other hand, the drug in the inter phase of the O/W structure would be released gradually into the blood, avoiding the irritation and injury caused by very high local concentrations of

DTX. The same target organs of both formulations may be explained by the tissue distribution study. The results of the tissue distribution study showed that the novel DLE did not change the distribution behavior compared with DS; therefore, the target organs were the same.

From the results of the toxicity study, it can be concluded that both DLE and DS exhibited toxic effects in hematology, serum biochemistry parameters and tissues, while the degree of toxicity in the DLE group was lower than that in the DS group. The major factor affecting the toxicity was DTX itself; therefore, there was no essential difference in the toxic effects of DLE and DS observed in this study. The lower toxic effects in the DLE group compared with the DS group were primarily because of the different states of DTX in the two formulations when exposed to blood and tissues. The DTX in DLE was embedded in the lipophilic groups of the phospholipid molecules and formed a mixture with phospholipids, and it was the phospholipid-DTX mixture that was in contact with the blood and tissues. However, the DTX in DS was present in an expansion micelle form, which was in direct contact with the blood and tissues. Hence, the toxic effects in the DS group were more serious than those in the DLE group.

CONCLUSION

In conclusion, the lipid emulsion delivery system has been applied to prepare a Tween 80-free formulation of DTX (DLE) for i.v. administration. The novel DLE, which has the advantages in safety and convenience during clinical administration, not only displays bioequivalent pharmacokinetics, similar tissue distribution behavior and antitumor activities to DS, but also exhibits lower toxic effects than DS at equivalent doses. Taking all the findings into consideration, the DLE investigated in this paper, is of great potential and valuable for clinical applications.

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