EXPERT OPINION

Preclinical evaluations of norcantharidin-loaded intravenous lipid microspheres with low toxicity

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Objectives: The aim of this study was to perform a systematic preclinical evaluation of norcantharidin (NCTD)-loaded intravenous lipid microspheres (NLM).

Research design and methods: Pharmacokinetics, biodistribution, antitumor efficacy and drug safety assessment (including acute toxicity, subchronic toxicity, hemolysis testing, intravenous stimulation and injection anaphylaxis) of NLM were carried out in comparison with the commercial product disodium norcantharidate injection (NI).

Results: The pharmacokinetics of NLM in rats was similar to that of NI, and a non-linear correlation was observed between AUC and dose. A comparable antitumor efficacy of NLM and NI was observed in mice inoculated with A549, BEL7402 and BCAP-37 cell lines. It was worth noting that the NLM produced a lower drug concentration in heart compared with NI, and significantly reduced the cardiac and renal toxicity. The LD_{50} of NLM was twice higher than that of NI. In NLM, over 80% of NCTD was loaded in the lipid phase or bound with phospholipids. Thus, NCTD was sequestered by direct contacting with body fluids and largely avoided distribution into tissues, consequently leading to significantly reduced cardiac and renal toxicity.

Conclusions: These preclinical results suggested that NLM could be a useful potential carrier for parenteral administration of NCTD, while providing a superior safety profile.

Keywords: antitumor efficacy, biodistribution, norcantharidin-loaded lipid microsphere, pharmacokinetics, reduced toxicity

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1. Introduction

Norcantharidin (NCTD), a demethylated analog of cantharidin, has been shown to be very effective against cancer, especially against primary hepatic carcinoma [1,2]. Previous reports have demonstrated that NCTD can inhibit tumor growth by inhibiting DNA replication, blocking cell cycle progression and inducing apoptosis via regulating Bcl-2 and p53 gene expression or via extracellular signal-regulated kinase and c-Jun-NH_2-terminal kinase signaling pathways [3-5]. Unlike the majority of anticancer drugs, the significant advantages of NCTD are the little myelosuppression and induction of leucocytosis [6,7]. In addition, NCTD is not a substrate for transmembrane efflux transporters, a well-known mechanism for inducing multidrug resistance for most cytotoxic drugs [3,8]. Therefore, NCTD is now more attractive for chemotherapy because of its significant antihepatoma activity and relatively low side effects.

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However, successful treatment with the conventional NCTD formulation remains difficult because of its high renal damage [9]. In addition, NCTD can induce multiple organ dysfunction syndromes (MODS), cardiac failure precedes renal and hepatic failure, following intraperitoneal injection in rats at a dose of 15 mg/kg [10]. The high cardiac and renal damage significantly limit the clinical usefulness of NCTD. Clinically, NCTD can be administrated by the oral or intravenous routes. At present, NCTD is still mostly given by injection of its sodium salt (5 mg/ml, 2 ml) at a pH of about 9.0. The high pH is a major cause of the irritation following intravenous injection. When administrated via oral route, NCTD has to be given in high dose to enhance its antitumor efficacy due to the low intestinal absorption, which might result in high gastrointestinal and urinary toxicity. Recently, chitosan nanoparticle was prepared to enhance intestinal absorption [11]. To further improve the antitumor effects and reduce toxicity, several NCTD sustained release preparations, such as poly(ε-caprolactone) microspheres and NCTD/poloxamer 407 injections have been designed for intrahepatic or intramuscular administration [12-14]. Recently, several investigations have focused on novel drug delivery systems (DDS) for intravenous administration, such as polymeric microspheres [15], microemulsions [16], liposomes and nanoparticles [17-19], which aim at the specific targeting of tumor, thus improving the efficacy and safety. However, most of these approaches have been clinically unsuccessful because of the complicated preparation procedure, the presence of an organic solvent and poor physicochemical stability during long-term storage.

In the past few decades, lipid microspheres (LM) has attracted great attention due to their well-known advantages such as physical stability, protection of labile drugs from degradation, ability to improve efficacy of lipophilic drugs and ease of production and scale up [20-22]. The therapeutic advantages of LM have been demonstrated by a decrease in drug toxicity for amphotericin B [23]. In addition, compared with normal tissue, the EPR effect of tumor offers an opportunity for an increased extravasation and retention of nanoparticles in the tumor interstitium [24]. Usually, the pore size of tumor vessels ranges from 100 to 780 nm [25]. By contrast, for most normal microvessels, the distance is less than 2 nm along the tight junctions in capillaries and less than 6 nm for postcapillary venules [26], except that the pore sizes of kidney and the two RES organs (liver and spleen) are 40 – 60 and 50 – 280 nm, respectively [27,28]. A Phase III trial of doxorubicin HCl-loaded lipid carriers confirmed that the lipid carriers could produce significantly reduced side effects due to reduced accumulation in healthy tissues without any EPR effect [29]. Therefore, the encapsulation of NCTD into LM might be a promising approach to reduce toxic effects.

In this study, a NLM (norcantharidin-loaded lipid microsphere) with a mean particle size ranging from 100 to 800 nm was proposed to encapsulate NCTD in the internal phase to avoid direct contact with body fluids and tissues. Consequently, a superior safety profile for parenteral administration of NCTD might be obtained while allowing administration of a higher 'dose density', thus potentially leading to greater antitumor efficacy.

The primary purpose of the present study was to systematically evaluate NLM in terms of pharmacokinetics, tissue distribution, antitumor activity and safety evaluation, compared with the commercial product NI (norcantharidate injection).

2. Materials and methods

2.1 Materials

NCTD was purchased from Jiangsu Kangxi Pharma Ltd. Co. (Jiangsu, China). Medium-chain triglyceride oil (MCT) and sodium oleate were supplied by Lipoid GmbH (Ludwigshafen, Germany). Egg phospholipids PL-100 M was obtained from QP Corp. (Tokyo, Japan). Long-chain triglyceride (LCT) was obtained from Tieling Beiya Pharmaceutical Co. (Tieling, China). Poloxamer 188 (Pluronic F68) was a kind gift from BASF AG (Ludwigshafen, Germany). Glycerol was kindly provided by Zhejiang Suichang Glycerol Plant (Zhejiang, China). NI (dissolved in double-distilled water to obtain 2 mg/ml NI prior to administration) was obtained from Heze Ruiying Pharma Ltd. Co. (Shandong, China). Ethyl acetate was obtained from Tianjin Concord Technology Ltd. Co. (Tianjin, China). Acetonitrile, methanol and formic acid were obtained from Dikma Co. (Richmond Hill, NY, USA) and egg albumin was obtained from Sigma-Aldrich Co., St. Louis, MO, USA. All other reagents were of analytical or chromatographic grade.

2.2 Preparation of NLM

NLM was prepared by homogenization. Briefly, the lipid phase consisted of MCT (7.5%, w/v), LCT (2.5%, w/v), PL-100M (3.6%, w/v) and NCTD (0.2%, w/v) was maintained at 70°C with stirring until a uniform system was obtained. An aqueous phase was prepared by dissolving F68 (0.4%, w/v), glycerin (2.5%, w/v) and sodium oleate (0.03%, w/v) in double-distilled water and heated at 75°C with stirring. Subsequently, the aqueous phase was added to the lipid phase using a high-speed shear mixer (ULTRA RURRAX®IKA® T18 basic, Germany) operated at 10,000 rpm for 5 min to obtain the primary emulsion. Then, the pH was adjusted to 7.2 using 1 M NaOH solution, and the volume was made up to 100% with double-distilled water. Then, the primary emulsion was passed through a high-pressure homogenizer (Niro Soavi NS10012k, Niro Soavi S.p.A., Via M. Da Erba, Italy) at 80 MPa for eight cycles to obtain the final emulsion. Finally, the preparation was gassed with N_2, sealed in vials and autoclaved at 121°C for 10 min to provide 2 mg/ml (calculated by NCTD) NLM for intravenous administration.

The prepared NLM exhibited a mean particle size of 167.4 ± 63.1 nm, and a zeta-potential of -31.6 mV. The relative percentage content and entrapment efficiency of NCTD were 101.8 and 84.9%, respectively.

2.3 Pharmacokinetic studies in rats

The pharmacokinetics of NLM in rats was investigated by comparison with the commercial product NI. Wistar rats weighting 180 – 220 g (Experimental Animal Center of Shenyang Pharmaceutical University, Shenyang, China) were allowed to acclimatize for 1 week then the animals were randomly divided into six groups of six rats (three males and three females per group) and fasted overnight with access to water *ad libitum* prior to the experiment. Three groups were treated with NLM by single i.v. dosing of 1.35, 2.7 and 4.05 mg/kg via the tail vein, while corresponding doses of NI were administered to the remaining three groups. The middle dose (2.7 mg/kg) was transferred from the clinical daily dose of NCTD (30 mg/day) by skin surface area conversion. Blood samples (about 0.3 ml) were collected into heparinized centrifuge tubes by retro-orbital puncture at predetermined time points (5, 15, 30 and 45 min, 1, 2, 4, 6, 8, 12 and 24 h). The samples were immediately centrifuged at 4000 rpm for 10 min and the plasma samples were collected and stored at -80°C until analysis.

2.4 Tissue distribution studies

Kunming strain mice weighting 18 – 22 g (Experimental Animal Center of Shenyang Pharmaceutical University) were divided into two groups (36 mice per group, half male and half female). NLM and NI were administered to the two groups at a dose of 3.9 mg/kg via the tail vein after dilution with 5% (w/w) glucose solution. Then 0.5, 1, 3, 6, 12 and 24 h after drug administration, blood samples were collected by retro-orbital puncture from the mice (six mice per group). Then, the mice were immediately sacrificed, and the heart, liver, spleen, lung, kidney, brain, stomach and intestine were excised, washed in normal saline and blotted dry with filter paper. The blood samples were centrifuged at 4000 rpm for 10 min to separate the plasma. All the biological samples were stored frozen at -80°C until analysis.

2.5 Plasma and tissue sample analysis

2.5.1 *Plasma sample preparation*

For this, 100 µl rat or dog plasma, 20 µl diphenhydramine solution (internal standard, 20 ng/ml) and 100 µl hydrochloric acid (0.1 M) were added to a 7 ml centrifuge tube, and vortexed for 1 min in a Liquid Fast Mixer (YKH-3, Liaoxi Medical Apparatus and Instruments Factory, China). Then, the mixture was extracted with 3 ml acetic ether and vortexed for 10 min. After centrifugation (FULGOR Refrigerated Centrifuge, GL-20B, Shanghai FULGOR Analytical Apparatus Ltd., Co., Shanghai, China) at 4000 rpm for 10 min, 2.5 ml supernatant was transferred to a 5 ml centrifuge tube and evaporated to dryness in a centrifugal concentrator (Labconco Corp., MO, USA) at 50°C. The residue was reconstituted with 100 µl methanol and centrifuged at 12,000 rpm for 10 min, and then 5 µl of the supernatant were injected into the UPLC-ESI-MS/MS system for analysis.

2.5.2 *Tissue sample preparation*

Each tissue sample was weighed accurately (0.1 g) and homogenized using a glass tissue homogenizer (DY89-II, Xinzhi Biological and Scientific Co., Ningbo, China) after addition of 0.5 ml physiologic saline. Then, 200 µl tissue homogenate, 20 µl IS solution (20 ng/ml) and 20 µl hydrochloric acid (1.0 M) were added to a 7 ml centrifuge tube, and treated similarly to the plasma samples and analyzed by UPLC-ESI-MS/MS.

2.5.3 *Sample determination by UPLC-ESI-MS/MS*

Liquid chromatography was performed using an ACQUITY™ UPLC system (Waters Corp., Milford, MA, USA) with an autosampler maintained at 4°C. The separation was carried out on an ACQUITY UPLC™ BEH C18 column (50 mm × 2.1 mm i.d., 1.7 µm; Waters Corp.) and the column temperature was maintained at 35°C. The analysis was carried out by gradient elution using water (A, containing 0.1% formic acid) and acetonitrile (B) as the mobile phase at a flow rate of 0.2 ml/min. The gradient conditions of the mobile phase were as follows: B was increased linearly from initially 20 to 60% during the first 1 min, and held for 1.2 min. After 2.5 min, the composition was reset to the initial composition and a 0.5 min re-equilibration time was allowed. The injection volume was 5 µl using the partial loop mode.

A Waters ACQUITY™ TQD triple quadrupole tandem mass spectrometer (Waters Corp., Manchester, UK) with an ESI interface was used for mass analysis. The ESI source was operated in positive ionization mode for NCTD and IS. The optimal ESI source parameters were as follows: capillary 1.6 kV, extractor 3.0 V and RF 0.1 V. The temperature of the source and desolvation was set at 100 and 400°C, respectively. Nitrogen was used as the desolvation gas (550 l/h) and cone gas (50 l/h) for nebulization. For collision-induced dissociation (CID), argon was used as the collision gas at a pressure of approximately 3.59×10^{-3} mbar. Quantification was carried out using the multiple reaction monitoring (MRM) mode. The cone voltage and collision energy for NCTD were 40 V and 10 eV, respectively, while those for the IS were 30 V and 15 eV, respectively. The fragmentation transitions for MRM were m/z 169.0 123.1 amu for NCTD, and m/z 256.2 166.9 amu for IS, with a scan time of 0.02 s per transition. All data collected in centroid mode were acquired using MassLynx™ NT4.1 software (Waters Corp.). Post-acquisition quantitative analyses were carried out using a QuanLynx™ program (Waters Corp.).

Validation of analytical method for NCTD showed that the chosen method was precise and accurate with a linear response range of 5 – 5000 ng/ml. The lower limit of quantification (LLOQ) was 5 ng/ml with a precision of 10.5%. The intra-day precision at three concentrations (10, 250 and 4000 ng/ml) was 2.6 – 13.6%, the inter-day precision was less than 12.1% and the accuracy was -11.6 to 9.1%. These values were within the limits (< 15%) specified for inter- and intra-precision, showing acceptable precision and

accuracy. The relative recoveries of NCTD from the plasma and tissue samples at three concentrations (10, 250 and 4000 ng/ml) were 60.66 – 67.69% and 64.16 – 68.69%, respectively.

2.5.4 Pharmacokinetics and statistical analysis

The pharmacokinetic results were processed using DAS 2.0 (Mathematical Pharmacology Professional Committee of China, Shanghai, China). The statistical differences between two preparations were assessed by Student's independent sample t-test and expressed as a one-way p-value. A value of $p < 0.05$ was considered statistically significant. The statistical analysis was performed using SPSS 11.5 (SPSS Inc., Chicago, IL, USA). All values were expressed as their mean ± S.D.

2.6 Pharmacodynamic studies

2.6.1 Animals and tumor cells

BALB/c-nu nude mice, purchased from the Experimental Animal Center of Medical Department of Beijing University (Beijing, China), were fed in a germ-free environment with free access to food and water. The A549 human pulmonary adenocarcinoma cell line and the BEL7402 hepatocellular carcinoma cell line were kindly provided by the Department of Pharmacology of the Cancer Institute of Chinese Academy of Medical Sciences (Beijing, China). The BCAP-37 human breast adenocarcinoma cell line was supplied by the First Department of Pharmacology in the Institute of Materia Medica (Chinese Academy of Medical Sciences).

2.6.2 *In vivo* antitumor efficacy in tumor-bearing mice

To estimate the antitumor effect of NCTD in xenograft tumor models, tumors were induced in BALB/c-nu nude mice by subcutaneous inoculation of approximately $1.5 \times 1.5 \times 1.5$ mm^3 human cancer cells in the right axillary region. The antitumor efficacy of NCTD against the A549 and BEL7402 lines were studied in male nude mice, while that of NCTD against the BCAP-37 line was studied in female nude mice.

At 11 – 15 days after tumor inoculation when the tumor volume (TV) reached approximately 100 mm^3, each treatment was started. Subsequently, the mice were randomly assigned to five groups according to the tumor size, with seven mice in each group (day 0). Three groups were given NLM at doses of 1.25 mg/kg (NLM-L), 2.5 mg/kg (NLM-M) and 5.0 mg/kg (NLM-H) via the tail vein. In the positive control (PC) group. NI was administered at a dose of 2.5 mg/kg in the same way. The NI and NLM were both diluted with drug-free LM to about 0.2 ml prior to administration. An equal volume of drug-free LM was injected into the tail vein of the remaining group mice as a negative control (NC) group. For each group, the administration was performed once a week for four weeks.

The activity against the three human cancer cell lines was investigated by the above method and the investigation was carried out twice to confirm the reproducibility and reliability for each cell line.

2.6.3 Antitumor efficacy assessment

The body weight was measured for each mouse on day 7, 14 and 24 after treatment started. The TVs were measured at day 4, 7, 11, 14, 19 and 24. The TV was calculated as follows: $TV = 1/2 \times L \times W^2$, where L was the long diameter and W was the short diameter. The values of L and W were determined by a Vernier caliper (01010157, Zhongmeideli Co., Zhengjiang, China) across two perpendicular diameters. The relative tumor volume (RTV) was calculated by the formula of $RTV = V_t/V_0$, where V_t referred to the mean measured TV at a predetermined time and V_0 referred to the mean TV at day 0. The tumor growth rate (T/C, %) was calculated by $T/C = RTV_t/RTV_c$, where RTV_t and RTV_c represented the RTV of the treatment groups and the NC group, respectively. On the 15th day after administration started, the mice were sacrificed by cervical dislocation, and tumors were collected and weighed. The tumor growth inhibition rate (TGI, %) was expressed by the following equation: $TGI = 1 - W_t/W_c$, where W_t and W_c indicated the mean tumor weight of the treatment groups and the NC group [30].

The antitumor activity was evaluated by three parameters, namely, RTV, T/C and TGI. An efficient antitumor activity was defined as T/C 40% and the TGI value was no less than 40%, as well as significant differences in TV or RTV between the treatment groups and the NC group.

2.7 Safety assessment

2.7.1 Acute toxicity

ICR mice, weighing 20 – 24 g, were kindly supplied by Shanghai Xipuer-Bikai Experimental Animal Co., Ltd (Shanghai, China) to estimate the acute toxicity of NLM and NI. The animals were divided into five groups of 20 mice each (equal numbers of male and female mice) and were, as closely as possible, matched for weight and size per group to reduce the variability of the mouse responses to the preparations. The NLM was diluted to 5% (w/w) with glucose solution to give a series of appropriate concentrations and injected via the tail vein at doses of 11.95, 14.22, 16.93, 20.16 and 24.00 mg/kg, respectively. The animals in all groups were carefully monitored for any toxic effects for 30 min after dosing and at 1, 2 and 4 h, and then daily thereafter until 14 days had passed completed. The weight of the surviving mice was recorded at day 3, 7 and 14. The acute toxicity of NI was investigated in a similar manner to the control group as described above, using doses of 5.00, 6.58, 8.66, 11.40 and 15.00 mg/kg. The median lethal dose (LD_{50}) and 95% confidence limit were determined using the Bliss method [31].

2.7.2 Subchronic toxicity study in rats

Ninety Wistar rats (200 ± 20 g) were randomly assigned to three groups with 30 rats in each group (equal numbers of males and females): the NLM group, the NI group and the NCTD-free LM group. NLM and NI were given to two groups at an i.v. dose of 2.7 mg/kg every day for 28 days. In

the same way, NCTD-free LM were given to the remaining group at a dose of 4.75 ml/kg. After 4 weeks, 20 rats in each group were sacrificed. The remaining 10 rats remained alive but were killed after a 10-day recovery period. The animals had their body weight and behavior monitored throughout the entire experimental period. At the predetermined time points mentioned (after 1 day, after 4 weeks and after the 10-day recovery period), blood samples collected in tubes containing ethylenediaminetetraacetic acid (EDTA) were subjected to hematological analysis, while the blood samples collected in tubes without additive were centrifuged to obtain serum for biochemical analysis. Moreover, all the sacrificed rats were subjected to systematic autopsies and organ samples (heart, kidneys, liver, lungs, spleen, brain, stomach, duodenum and the tissue at the injection site) were fixed in 10% formalin for histopathological examination.

The hematological parameters, the red blood cell count (RBC), hematocrit (HCT), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular volume (MCV), white blood cell count (WBC), WBC differential count (DC), blood platelet count (PLT) and coagulation time (CT) were determined using an automatic blood analyzer. Serum samples were analyzed using an automatic biochemical analyzer (HITACHI-7060, Japan) to determine levels of aspartate transaminase (AST), alkaline phosphatase (ALP), alanine transaminase (ALT), blood urea nitrogen (BUN), total protein (TP), albumin (ALB), total bilirubin (TBIL), glucose (GLU), triglyceride (TG), total cholesterol (TCHO), creatinine kinase (CK), gamma-glutamyl transferase (γ-GGT), K, Na and Cl.

2.7.3 Hemolysis testing

Hemolysis experiments were performed using rabbit blood [32]. Briefly, 100 ml rabbit blood was obtained from the arteria cruralis. After removing the fibrinogen by shaking with glass beads for 10 min, 100 ml 0.9% physiologic saline was added, and the supernatant was removed following centrifugation at 1500 rpm for 15 min. The obtained erythrocytes were washed three times (centrifugation followed by redispersion) with 0.9% physiologic saline. Finally, a suitable amount of 0.9% physiologic saline was added to the erythrocyte pellets to give a 2% erythrocyte standard dispersion.

NLM and NI were diluted with 0.9% physiologic saline to obtain a final concentration of 0.3 mg/ml. Different amounts of diluted NLM and NI (0.3 mg/ml) with volumes of 0.7, 0.9, 1.1, 1.3 and 1.5 ml were added to tubes each containing 2.5 ml of 2% erythrocyte standard dispersion. Then suitable amounts of 0.9% physiologic saline were added to every tube to obtain a total volume of 6 ml. The negative control was prepared by addition of drug-free LM instead of the preparation. Then, 3.5 ml water, as the positive control, was added to 2.5 ml 2% erythrocyte standard dispersion. After vortexing, the tubes were incubated at 37°C and then after 20 and 40 min, 1, 2 and 3 h, the tubes were centrifuged at 1000 rpm for 10 min to obtain the supernatant. When the supernatant appeared to be red, it was concluded that hemolysis had occurred. However, a clear supernatant indicated no hemolysis.

2.7.4 Intravenous irritation assessment

Twelve rabbits weighing 2.13 ± 0.11 kg (Beijing Weitonglihua Experimental Animal Co., Ltd, Beijing, China) were randomly divided into four groups, each with three animals per group. NLM and NI were separately injected into the marginal ear vein of two groups (five ears in each group) at a daily dose of 0.5 mg/ml for 2 days. The other two groups received an equivalent volume of 0.9% saline and drug-free LM in a similar way as the control. Paradoxical reactions at the injection site were recorded at 24, 48 and 72 h after the last injection. The rabbits were sacrificed by exsanguination at 24 h after the last administration, and then the vascular tissues at the injection site were dissected and preserved in 10% formaldehyde for histological examination.

2.7.5 Injection anaphylaxis study

2.7.5.1 Active anaphylaxis

Thirty-six Hartley guinea pigs (weight 250 – 280 g) were divided into six groups (n = 6, equal numbers of males and females). Normal saline solution was given to the NC group, while 1% egg albumin solution was given to the PC group. The remaining four groups received injections of NLM and NI at sensitizing doses of 0.39 and 0.195 mg/kg. All the groups received intraperitoneal injections every other day, three times. On the 12th day after the last injection, every guinea pig was given a challenge dose intravenously, which was three times the sensitizing dose. The animals were monitored for 3 h in order to record any hypersensitivity reaction after the challenge injection.

2.7.5.2 Passive cutaneous anaphylaxis

Firstly, rats weighing 180 – 220 g were randomly assigned to six groups (n = 4) to prepare antiserum: i) NC group (0.9% saline injection); ii) PC group (1% egg albumin solution); iii) NLM (0.45 mg/kg); iv) NLM (0.225 mg/kg); v) NI (0.45 mg/kg) and vi) NI (0.225 mg/kg). After injection of the corresponding analyte, each group was given a total volume of 1.0 ml 5% $Al(OH)_3$ in normal saline subcutaneously. All the groups received similar administrations every other day, three times. Then, 12 days after the last sensitization, blood was obtained by retro-orbital puncture, and the serum was separated by centrifugation at 1500 *g* for 10 min, and stored at -20°C.

Then, another 48 male rats were divided into six groups, as described above and 0.1 ml 16-, 4- and 2-fold diluted corresponding antiserum was injected intracutaneously at six zygomorphic sites on the backbone. Then, 48 h after sensitization, 1 ml challenge solution containing 0.5 ml 1% Evans blue solution was given to the groups intravenously, at the same dose as the sensitizing dose. After 30 min, the rats were sacrificed, and the dorsal skin was removed to determine the diameter of the blue spot.

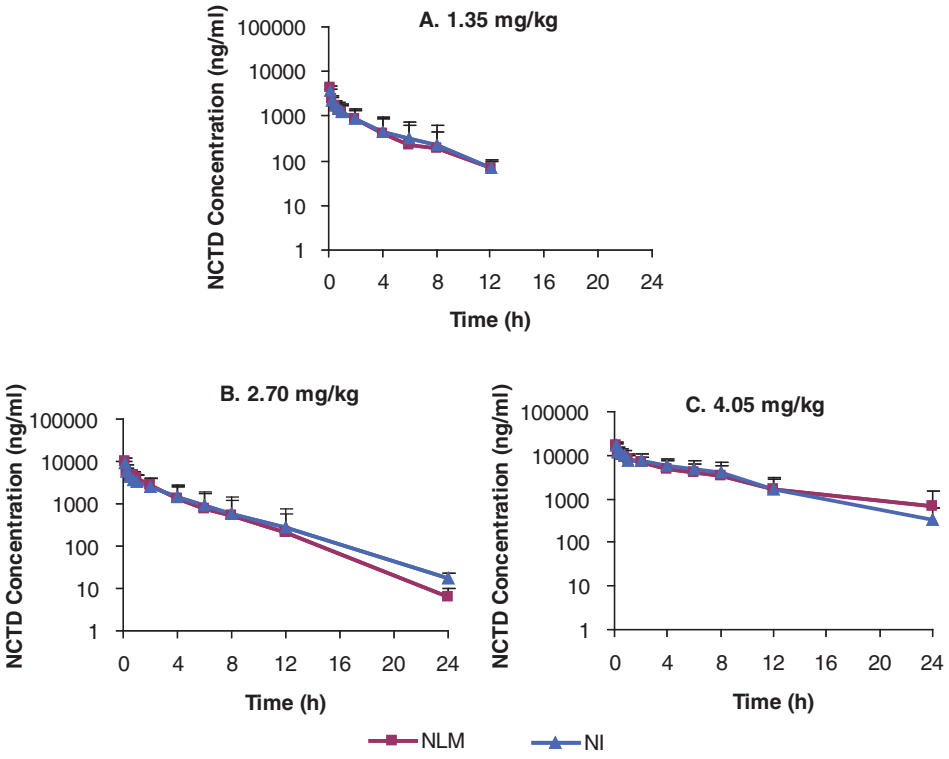

Figure 1. Mean plasma concentration–time profiles of NCTD after intravenous administration of NLM and NI to rats at doses of (A) 1.35 mg/kg, (B) 2.70 mg/kg and (C) 4.05 mg/kg. Each point represents the mean ± S.D. (n = 6).

All animal experiments were conducted in compliance with the Guidelines for the Care and Use of Laboratory Animals. The protocol was approved by the University Ethics Committee for the use of laboratory animals.

3. Results

3.1 Pharmacokinetic study in rats

The pharmacokinetic investigations of NLM and NI were carried out by determining the concentration of NCTD in rat plasma after a single i.v. administration at a dose of 1.35, 2.7 and 4.05 mg/kg. The mean plasma concentration–time profiles at different doses are shown in **Figure 1**. The profiles were best fitted to a two-compartment model with a weighting factor of $1/c^2$. The main pharmacokinetic parameters were calculated by the statistical moment method and are listed in **Table 1**. There were no significant differences ($p > 0.05$) in pharmacokinetic parameters between the two study groups at the same dose level. Furthermore, according to the Food and Drug Administration (FDA) guidance, NLM was bioequivalent to NI in rats, with 90% confidence intervals for the area under the curve (AUC) and C_{max} ratios ranging from 0.80 to 1.25.

For both of NLM and NI, the dose-ratio was 3:2:1, but the corresponding AUC_{0-t} ratio was about 10:2:1. NCTD displayed a linear elimination at the dose range of 1.35 – 2.07 mg/kg. However, there was a significant increase in the AUC_{0-t} and $t_{1/2}$, and a decrease in clearance (CL) on increasing the dose from 1.35 to 4.05 mg/kg ($p < 0.05$) for the two preparations. The significantly slower elimination indicated that a certain degree of saturation of the mechanism of NCTD removal from blood occurred at higher doses.

3.2 Tissue distribution studies

The plasma and tissue NCTD concentrations versus time after i.v. administration of NLM and NI at a dose of 3.9 mg/kg to mice are shown in **Figure 2**. Since there was no NCTD to be detected in brain after the administration, the brain results are not provided. The AUC values in different tissues of the two formulations are reported in **Figure 3**. Thirty minutes after administration, the NCTD concentration in tissues was in the order of kidney > blood > lung > liver > spleen > heart > brain. There was no difference in tissue distribution behavior between NLM and NI, except for the heart. The drug level in the heart for the LM group was always lower than that of NI at any time during the study period. The NCTD AUC_{0-t} in the heart after administration of NLM was less than that of NI (**Figure 3**).

3.3 Antitumor efficacy *in vivo*

The antitumor activities of NLM against A549, BEL7402 and BCAP-37 in tumor-bearing nude mice were investigated and compared with NI. **Figure 4** shows the growth curves of tumors in BALB/c-nu nude mice after intravenous administration of NC, NI (2.5 mg/kg), NLM-L (1.25 mg/kg), NLM-M

Table 1. Pharmacokinetic parameters of NCTD after intravenous administration of NLM and NI to rats at doses of 1.35, 2.70 and 4.05 mg/kg (mean ± S.D., n = 6).

Parameter	Unit	NLM			NI		
		1.35 mg/kg	2.70 mg/kg	4.05 mg/kg	1.35 mg/kg	2.70 mg/kg	4.05 mg/kg
AUC_{0-t}	μg/L h	6479.7 ± 5256.4	28410.8 ± 18286.8	70287.7 ± 30880.6	7258.9 ± 6052.7	18454.1 ± 13467.0	69310.7 ± 23569.7
$AUC_{0-\infty}$	μg/L h	6558.0 ± 5317.6	34663.3 ± 24805.3	82640.0 ± 46130.8	7808.7 ± 7200.5	18735.0 ± 13874.0	73186.4 ± 26219.2
MRT	h	2.19 ± 1.93	3.75 ± 2.52	5.64 ± 2.51	2.75 ± 2.38	2.71 ± 1.99	5.38 ± 1.60
$t_{1/2}$	h	1.64 ± 1.13	4.32 ± 3.49	6.35 ± 5.36	2.51 ± 2.79	2.22 ± 1.33	5.32 ± 1.78
CL	l/h/kg	0.30 ± 0.15	0.13 ± 0.10	0.07 ± 0.04	0.26 ± 0.14	0.22 ± 0.14	0.06 ± 0.03
V_{ss}	l/kg	0.51 ± 0.09	0.46 ± 0.10	0.40 ± 0.14	0.60 ± 0.17	0.53 ± 0.19	0.45 ± 0.12
C_{max}	μg/l	4224.7 ± 521.9	9594.7 ± 1924.3	16906.0 ± 1402.4	3642.2 ± 346.6	8762.8 ± 1602.0	16638.5 ± 3517.4

AUC: Area under plasma concentration–time curve; C_{max}: Maximum concentration; CL: Clearance; MRT: Mean residence time; NI: Norcantharidate injection; NLM: Norcantharidin-loaded lipid microsphere; $t_{1/2}$: Elimination half-life; V_{ss}: Apparent volume of distribution at steady state.

(2.5 mg/kg) and NLM-H (5.0 mg/kg). The values of RTV, T/C and TGI for each group are listed in **Table 2**. The TVs in NC group increased rapidly, indicating that drug-free LM had no measurable effect on tumor growth. As shown in **Table 2**, all the NLM and NI groups exhibited marked anti-tumor activity against A549, BEL7402 and BCAP-37 with a T/C less than 40% and a TGI higher than 40%, especially for treating BEL7402 tumors with a T/C lower than 20% in the NLM-H group. Moreover, moderate activity in the three cell lines without any significant difference ($p > 0.05$) was observed between NLM and NI groups at the same dose level (2.5 mg/kg).

3.4 Intravenous injection safety testing

3.4.1 Acute single dose toxicity

Single dose toxicity studies of NLM and NI in healthy mice were carried out to compare the toxicity of the two formulations. The mortality and general behavior adverse effects changed in a dose-dependent manner (**Figure 5**). For the NI groups, toxic symptoms (hypoactivity and attenuated response to external stimuli) were observed in some mice 4 h after administration at doses of 11.40 and 15.00 mg/kg. Also, the mice with the toxic symptoms had all died by the second day of administration. The LD_{50} value and 95% confidence limit of NI for female mice and male mice administered i.v. were calculated to be 10.10 (8.33 – 13.10) and 8.93 (6.92 – 11.77) mg/kg, respectively.

In the case of the NLM groups, hypoactivity and attenuated response to external stimuli were observed immediately after administration at a dose of 20.16 and 24.00 mg/kg. The toxic symptoms of the surviving mice disappeared within 16 min to 1 h after injection. The calculated LD_{50} was 15.67 mg/kg (95% confidence intervals: 13.61 – 17.58 mg/kg) for female mice and the LD_{50} was 16.64 mg/kg (95% confidence intervals: 15.14 – 18.25 mg/kg) for male mice, which was equivalent to twice that of NI. Autopsies on all the treated animals revealed no macroscopic changes in major organs, such as heart, liver, spleen, lung and kidney.

3.4.2 Subchronic toxicity study in rats

No significant differences in body weight changes were noted between the NLM and NI groups, and the NLM group and NCTD-free LM group at any time. Furthermore, there were no animal deaths in any group during the entire study period.

The hematology examination results indicated that all the parameters remained within normal limits throughout the treatment period, except that the WBC of the NCTD-treated groups ($18.5 \pm 3.4 \times 10^9$ l^{-1} for NLM group and $15.6 \pm 2.4 \times 10^9$ l^{-1} for NI group) were both slightly higher than that of the drug-free group ($11.8 \pm 2.4 \times 10^9$ l^{-1}) after a 4-week treatment. During the recovery period, the WBC in each group was restored to the normal level. Serum biochemistry analysis indicated that the administration of NLM did not cause any significant changes in any biochemistry parameters during the entire experiment.

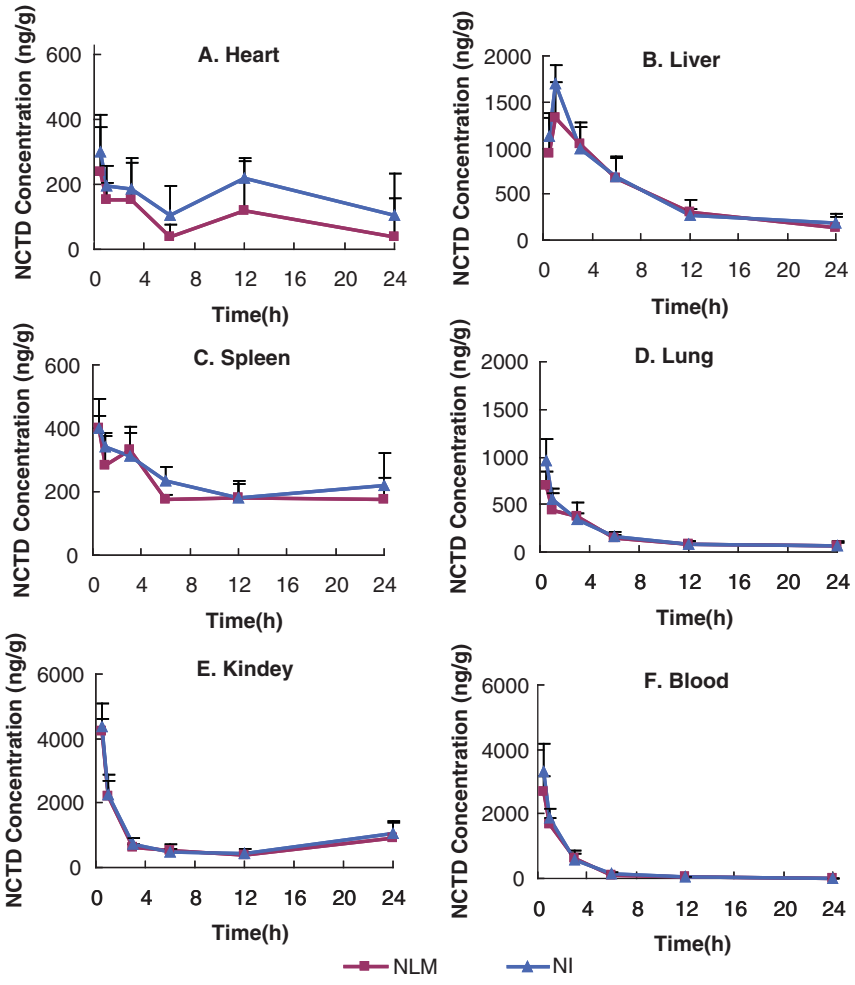

Figure 2. Mean concentration–time profiles of NCTD in (A) heart, (B) spleen, (C) liver, (D) lung, (E) kidney and (F) plasma after intravenous administration of NLM and NI to mice at a dose of 3.9 mg/kg. Each point represents the mean ± S.D. (n = 6).

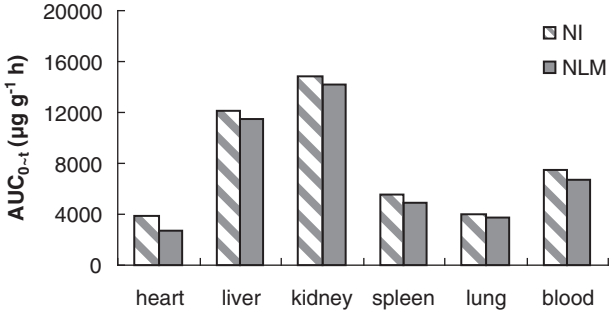

Figure 3. AUC_{0-t} values in different tissues after intravenous administration of NLM and NI to mice at a dose of 3.9 mg/kg (n = 6).

A histopathology examination was carried out to study the organ damage induced by NLM or NI. After a 4-week treatment, no obvious damage was observed on liver, lungs, spleen, brain, stomach, duodenum and the tissue at the injection site. It was worth noting that the administration of NI caused significant cardiac and renal toxicity, which could be characterized by fragmentation of the myocardium, myocardial edema, kidney necrosis and cloudy swelling of the renal tubules (**Figure 6**). The incidence of cardiac toxicity was 66.7% (20/30), while that of nephrotoxicity was 73.3% (22/30). However, in the case of the NLM group, there was no obvious damage to the heart. Also, a relatively lower renal toxicity was observed with an incidence of 33.3% (10/30) (**Figure 7**). After the recovery period, the renal damage disappeared and there was no obvious organ damage to be observed in the NLM group. However, serious damage to the heart was still observed in the NI group (**Figure 6**).

3.4.3 Hemolysis testing

Complete hemolysis was observed in tubes of the positive control at 20 min, the solution was red clear and no erythrocytes survived at the bottom of the tubes. During the 3 h observation period, the tubes in the NC group, NLM groups and NI groups appeared to be clear, with the erythrocytes precipitated

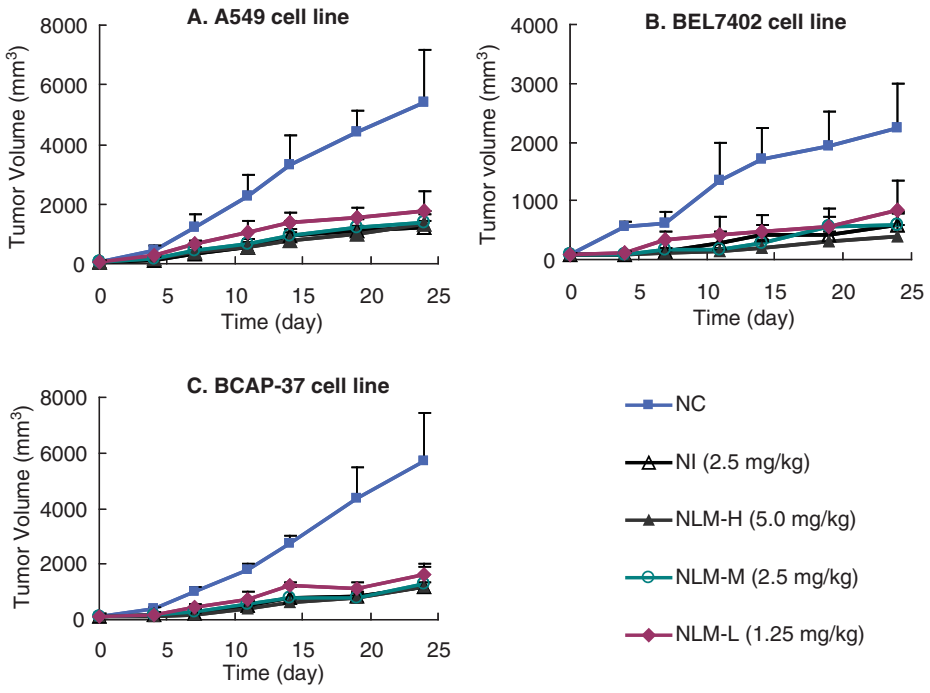

Figure 4. The tumor growth curves of tumor-bearing mice after intravenous administration of NC, NI (2.5 mg/kg), NLM-L (1.25 mg/kg), NLM-M (2.5 mg/kg) and NLM-H (5.0 mg/kg). The antitumor efficacy of NCTD against (**A**) A549 cell line; (**B**) BEL7402 cell line and (**C**) BCAP-37 cell line were studied in BALB/c-nu nude mice. Each point represents the mean ± S.D. (n = 7).

Table 2. The antitumor activities of each group against A549, BEL7402 and BCAP-37 in tumor-bearing mice 24 days after administration (mean ± S.D., n = 7).

	NC	NI (2.5 mg/kg)	NLM-H (5.0 mg/kg)	NLM-M (2.5 mg/kg)	NLM-L (1.25 mg/kg)
A549 cell line					
RTV*	77.30 ± 31.00	18.19 ± 6.97	19.94 ± 8.04	19.48 ± 6.90	26.07 ± 12.07
T/C (%)	–	23.5	25.8	25.2	33.7
TGI (%)	–	68.7	73.1	65.6	39.2
BEL7402 cell line					
RTV*	30.10 ± 14.50	7.57 ± 2.68	5.16 ± 1.93	8.52 ± 5.15	11.74 ± 9.48
T/C (%)	–	25.2	17.2	28.4	39.1
TGI (%)	–	65.9	70.4	64.2	58.9
BCAP-37 cell line					
RTV*	50.08 ± 17.13	10.29 ± 3.97	11.16 ± 4.05	11.31 ± 6.89	15.65 ± 5.89
T/C (%)	–	20.6	23.3	22.6	31.3
TGI (%)	–	65.9	70.7	57.3	56.3

NI: Norcantharidate injection; NLM: Norcantharidin-loaded lipid microsphere; RTV: Relative tumor volume; T/C: Tumor growth rate; TGI: Tumor growth inhibition.
*$p < 0.01$ compared with the NC group.

at the bottom. These experimental phenomena showed that both NLM and NI at different concentrations did not cause any hemolysis or erythrocyte agglutination at 37°C.

3.4.4 *Intravenous irritation assessment*

After a 3-day administration, no erythema or edema were observed at the injection sites of all the groups. In addition, the histopathologic examination of the rabbit ear-border vein indicated that that there was no angiectasia and vascular congestion in blood vessels at or away from the site of injection in all groups during the acute reaction period. Furthermore, no thrombus or hyperplasia of endothelial cells appeared in the blood vessels. There was slight swelling and hemorrhage at the injection sites and the surrounding tissues, but no inflammatory cell infiltrate. The histopathologic examination results for the ears of the rabbits given NLM were similar to those of the NI group. During the convalescent period, the histopathologic examination of the rabbit ear-border veins

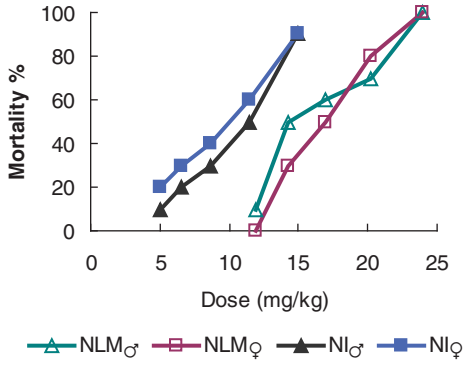

Figure 5. Mortality dose–response relationships for NLM and NI in male (♂) and female (♀) mice after intravenous administration.

showed no obvious change. The phenomena described above were similar in all the groups, indicating that no obvious intravenous irritation was caused by both NLM and NI.

3.4.5 *Hypersensitivity reaction*

In the active anaphylaxis study, all the guinea pigs in the PC group exhibited significant hypersensitivity symptoms including dyspnea, gait instability, Cheyne-Stokes respiration and convulsion and then died. However, there were no obvious anaphylaxis symptoms, such as nose scratching, sneezing, erect hair, twitching, dyspnea and convulsions in the NC groups, in the NLM and NI groups after the last challenge. In the passive cutaneous anaphylaxis study, no blue spot appeared in the NLM and NI groups. Therefore, it is clear that the intravenous administration of NLM is safe and does not cause hypersensitivity reactions.

4. Discussion

As indicated in **Table 1**, the dose of NCTD in NLM increased from 1.35 to 4.05 mg/kg, the plasma AUC increased from 6479.7 to 70287.7 μg/L h and the $t_{1/2}$ was prolonged from 1.64 to 6.35 h. A threefold increase in the dose of NCTD resulted in a non-linear approximately 10-fold increase in the plasma AUC. The calculated terminal half-life ($t_{1/2}$) fell over-proportionally with the increase in dose. Similar results were also observed for the NI. These results indicate non-linear pharmacokinetic behavior of NCTD in rats. Following dose escalation, the over-proportionally increased AUC might contribute to the enzyme equilibrium processes of elimination and distribution of NCTD. In addition, a previous report also found that several metabolites in rats after treatment with NCTD exhibited low correlations between concentration and dosage [33]. It might be inferred that NCTD exhibited a narrow therapeutic window. These results suggested that the dose of NCTD should be controlled rigorously during clinical administration.

To better understand the deposition of NLM *in vivo*, a comparative tissue distribution investigation was carried out between NLM and NI. After i.v. administration, the two preparations showed no difference between the NCTD concentrations in different mouse tissues, including liver, spleen, lung, kidney and brain, except for the heart. NLM produced a lower NCTD concentration and AUC in the heart than NI. As reported previously, the administration of NCTD can cause MODS, especially involving cardiac dysfunction [10]. Since a relatively low NCTD concentration was found in the heart after administration of NLM compared with NI, NLM might have a reduced toxicity in the heart. As expected, an increased LD_{50} was observed by incorporating NCTD in a LM, which indicated that NLM exhibited a lower toxicity than NI.

To further confirm the reduced toxicity of NLM compared with NI, necropsy and histopathology examinations were performed after 4-week continuous administration to rats. Significant damage to the heart and kidney in the NI group was observed after the 4-week administration period. Also, significant heart damage and slight renal damage were still observed after a 10-day recovery period. This indicated that NI could cause irreversible damage to the heart. However, NLM produced no obvious damage to the heart and the mild toxic effect on the kidney, which disappeared after the recovery period. Based on the above observations, the NLM had a significantly lower toxic effect on heart than the commercial formulation NI. These results confirmed the potential advantage of the NLM over NI in reducing the cardiac and renal toxicity. Meanwhile, as indicated in the antitumor activity investigation, NLM displayed significant antitumor activities against A549, BEL7402 and BCAP-37, similar to the commercial preparation. Also, BEL7402 was found to be the most sensitive cell line to NCTD, followed by BCAP-37 and then A549. This indicated that NLM might be an excellent treatment for liver cancer in clinical situations. Moreover, the rats after being treated with high-dose NLM (5.0 mg/kg) did not show any appreciable loss in body weight over the entire study period. In the safety test, it can be concluded that NLM, which produced no irritation, hemolysis or hypersensitivity, was as safe as the commercial product NI. These results indicate that NLM reduces toxicity with analogous antitumor activity of NI.

The significantly reduced toxicity of NLM can be ascribed to the unique structure and characteristics of LM. Generally, LMs are rapidly taken up by the RES in the liver, spleen and lung after i.v. injection [34,35]. Oil globules between 2 and 3 μm in size appear to be preferentially taken up by the liver [36], while smaller particles (< 0.2 μm) could avoid trapping by the RES [37,38]. The NLM exhibited a mean particle size of 167.4 ± 63.1 nm, which was smaller than 200 nm. Therefore, NLM could escape the capture by the RES of liver, spleen and lung, leading to comparable AUCs in the above tissues with those of NI (**Figure 3**). However, the lower NCTD concentration in the heart with less RES may be attributed to

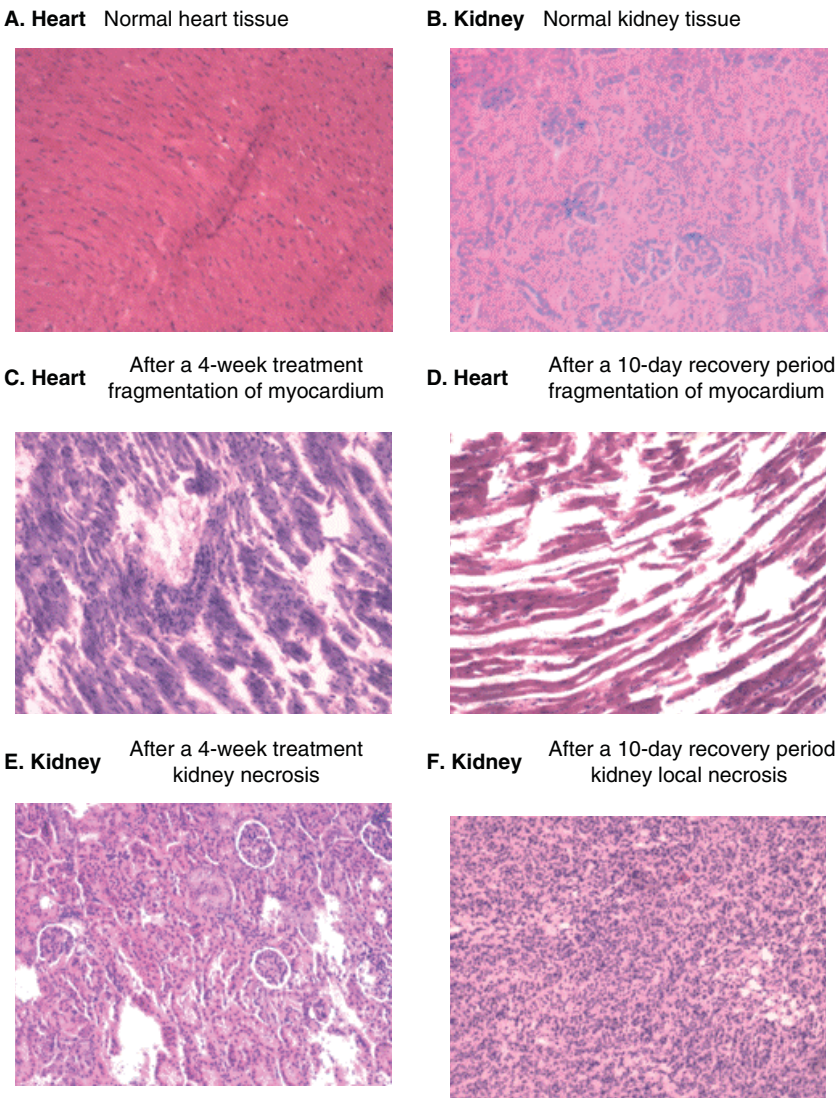

Figure 6. Histopathology examinations of heart and kidney specimens from NI-treated (4 weeks of continuous treatment with NI at a dose of 2.7 mg/kg/day) rats. (**A**) Normal heart section; (**B**) normal kidney section; (**C**) heart sections in NI-treated group (fragmentation of myocardium was observed); (**D**) heart sections in NI-treated group after a 10-day recovery period (fragmentation of myocardium was observed); (**E**) kidney sections in NI-treated group (kidney necrosis was observed) and (**F**) kidney sections in NI-treated group after a 10-day recovery period (local kidney necrosis was observed).

the structure of the LM. In NLM, over 80% of the NCTD was loaded in the oil and water interfacial surface [39]. The incorporated drug was dissolved in the soybean lipid phase of the oil-in-water emulsion droplets or bound with phospholipids. After intravenous administration, many plasma proteins associate with the emulsion droplet surfaces. The lipid exchange between lipid-based emulsion and apolipoprotein can lead to disruption of the LM with concomitant release of incorporated and lipid-bound drug [40]. NCTD exists in the blood as equilibrium among free sodium of NCTD in blood, protein-bound NCTD, soybean oil-dissolved NCTD and lipid-bound NCTD. Only the free sodium of NCTD can be transported efficiently across the blood vessels and into tissues. The incorporated drug or lipid-bound NCTD is sequestered by direct contact with body fluids and largely avoids the distribution into the heart. As a result, relatively low localization of NCTD in heart occurs after treatment with NLM. Subsequently, the NLM group displayed a lower cardiac toxicity compared with the NI group.

The reduced nephrotoxicity of NLM might be explained as follows. As shown in the results, the nephrotoxicity of NCTD was mainly characterized by cloudy swelling of renal tubules, tubular necrosis and kidney necrosis. Since NCTD is mainly excreted via the urine, the reduced toxicity on renal tubules might be attributed to the reduced NCTD concentration in the original urine. The formation of original urine is a

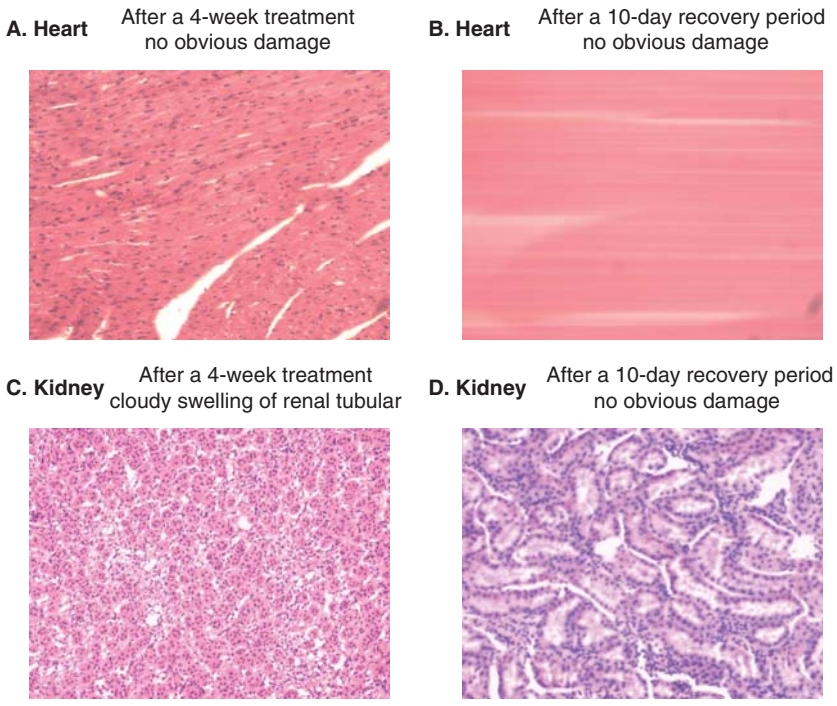

Figure 7. Histopathology examinations of heart and kidney specimens from NLM-treated (4 weeks of continuous treatment with NLM at a dose of 2.7 mg/kg/day) rats. (**A**) Heart sections in NLM-treated group (no obvious damage was observed); (**B**) heart sections in NLM-treated group after a 10-day recovery period (no obvious damage was observed); (**C**) kidney sections in NLM-treated group (cloudy swelling of renal tubular was observed) and (**D**) kidney sections in NLM-treated group after a 10-day recovery period (no obvious damage was observed).

filtration process by which the blood that passes through the glomerulus is filtered out. However, the pore size of the fenestrated endothelium of the glomerulus is 40 – 60 nm [27]. Therefore, only small molecules can pass through the glomerulus. After i.v. administration of NLM, part of the NCTD remains associated with LM, which prevents NCTD from passing through the glomerulus. A lower NCTD concentration in original urine might be obtained after treatment with NLM compared with NI. As a result, the renal tubule toxicity is reduced. However, a study of the mechanism for the reduced toxicity is required and this is in progress.

5. Conclusions

In conclusion, lipid microsphere has been found to be a suitable DDS for NCTD. The novel NLM displays similar pharmacokinetic and tissue distribution, and comparable antitumor efficacy with NI, while significantly reduces the toxic effects, especially cardiac and renal toxicity. These preclinical results suggest that NLM could be an effective and low-toxic parenteral carrier for NCTD delivery, while allowing a higher 'dose density', thus, potentially leading to greater antitumor efficacy.

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Declaration of interest

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