Drug Development and Industrial Pharmacy

ISSN: 0363-9045 (Print) 1520-5762 (Online) Journal homepage: http://www.tandfonline.com/loi/iddi20

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To cite this article: Dong Liu, Jing Xing, Fei Xiong, Fang Yang & Ning Gu (2016): Preparation and in vivo safety evaluations of antileukemic homoharringtonine-loaded PEGylated liposomes, Drug Development and Industrial Pharmacy, DOI: 10.1080/03639045.2016.1275670

To link to this article: http://dx.doi.org/10.1080/03639045.2016.1275670

Preparation and *in vivo* safety evaluations of antileukemic homoharringtonine-loaded PEGylated liposomes

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Abstract

In order to improve the *in vivo* safety and specific delivery efficiency of the antileukemic homoharringtonine (HHT) at the targets, the long-circulating PEGylated liposomes loaded with HHT (LCLipo-HHT) were prepared. Their physical characteristics, *in vitro* drug release, *in vivo* pharmacokinetic properties and elementary toxicity were evaluated. The mean diameter of the prepared LCLipo-HHT is 75.6 ± 3.2 nm and the zeta potential is -16.9 ± 2.5 mV. The entrapment efficiency (EE) of HHT in the liposomes is 69.5 ± 1.7%. In pharmacokinetic experiments, an increased plasma concentration as well as blood circulation time were obtained when distearoyl phosphoethanolamine (DSPE)-PEG 2000 lipid was added in the formulation, which results in enhancing drug delivery efficiency. Hemolysis test, vascular irritation test, and acute toxicity test were used to demonstrate toxicity of LCLipo-HHT. Compared with clinical HHT injection dosage, LCLipo-HHT indicated no vascular irritation, good hemocompatibility，as well as much better safety. Therefore, the prepared LCLipo-HHT can be used as a promising anticancer formulation for antileukemic therapy in the future.

Keywords: Homoharringtonine; PEGylated liposome; long-circulation; leukaemia; safety.

Introduction

Homoharringtonine (HHT) is a cytotoxic alkaloid with potent myelosuppressive activity isolated from the evergreen tree cephalotaxus harringtonia[1,2]. HHT has been used widely in the treatment for chronic myeloid leukemia (CML), acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) due to the unique mechanism of action of preventing the initial elongation step of protein synthesis[3]. Moreover, the interest in HHT for CML has been encouraged by positive results in patients' refractory to tyrosine kinase inhibitors in recent years[4-8]. Until now, it has been wildly used in China for treatment of leukemia based on the lyophilized powder injection dosage forms[5]. However, the clinical application of HHT is still limited owing to several obstacles resulting from the dosage form, such as its adverse reaction of myelosuppression, cardiac toxicity as well as hepatic impairment[2]. Therefore, it is desirable to explore novel formulations of HHT that overcome these disadvantages.

Liposomes have shown an increasing importance in the development of drug delivery systems[9]. It can not only improve the drug stability and solubility, enhance tumor accumulation, but also decrease the drug toxicity. Dozens of liposomal formulations have been approved for clinical use and a number of liposomal formulations are in the pipeline from concept to clinical application, which indicates that the liposomes used as drug carriers may be feasible for clinical applications[10,12]. Nevertheless, because of the adsorption of plasma protein to the phospholipid membrane of liposomes, they are often rapidly opsonized and cleared by the mononuclear phagocytic system (MPS)[13,14]. PEGylation is one of the most promising strategies that have been extensively studied for increasing the blood circulation time of nanoparticulate drug delivery systems[15-17]. As well documented in the literature, liposomes modified with polyethyleneglycol (PEG) have significantly extended blood circulation time in the body because of the protective layer that reduces MPS uptake [18]. Consequently, long-circulating liposomes with enhanced accumulation in tumor tissues *via* passive targeting can enhance the anticancer efficacy, meantime, can reduce the toxicity profiles of chemotherapeutic agents[19,20]. Doxil®, for instance, the first nanomedicine approved by Food and Drug Administration (FDA) is based on PEGylated liposomes[21].

Previously, we have prepared PEGylated homoharringtonine (HHT) encapsulated liposomes (LCLipo-HHT). The pharmacodynamics evaluation results show that compared with conventional liposomes containing HHT (Lipo-HHT) and HHT injection, LCLipo-HHT indicates a higher association with cytotoxicity against multiple myeloma (MM) RPMI8226 cells[22]. However, in order to further optimize the LCLipo-HHT dosage form and understand the pharmacokinetics as well as safety characteristics, in this study, and the optimized HHT-loaded PEGylated liposomes (LCLipo-HHT) was constructed. The physicochemical properties, blood kinetic profiles, bio-distribution of LCLipo-HHT were evaluated. The safety of LCLipo-HHT was also investigated.

Materials and methods

Materials

Homoharringtonine (HHT) (purity >98%) was purchased from Dalian Meilun Biotech CO., Ltd. (Dalian, China). HHT and harringtonine (HT) standard (purity > 99%) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). HHT injection (Inj-HHT) was purchased from Diesel Biological Pharmaceuticals CO., Ltd. (Xi'an, China). Phosphatidylcholine (Lipoid E80, egg yolk lecithin with 80-85% of phosphatidylcholine) was purchased from Lipoid GmbH (Ludwigshafen, Germany). Distearoyl phosphoethanolamine-Polyethylene glycol 2000 (DSPE-PEG 2000) was obtained as a gift sample from Southeast Pharmaceuticals CO., Ltd. (Suzhou, China). Cholesterol was purchased from BASF (China) Co., Ltd. (Shanghai, China). Other chemicals used were of analytical grade. BALB/c mice (male, 4 weeks old, 20-25 g) were purchased from the Experimental Animal Center of Southeast University (Jiangsu, China). The animals used for the experiment were treated according to the protocols evaluated and approved by the ethical committee of Southeast University (Nanjing, China).

Formulation of conventional and PEGylated liposomes

The ethanol injection and sonication method was used to prepare conventional and PEGylated liposomes[22]. Briefly, the PEGylated liposomes formulation was composed of 1 mg of HHT, 15 mg of phosphatidylcholine, 0.5 mg of cholesterol, and 2 mg of DSPE-PEG2000. The prescribed ingredients were synchronously dissolved in 2 mL of ethanol with ultrasound. Then this solution was added in 10 mL double distilled water slowly drop by drop with vortex

to get the mixture. The water bath temperature was maintained at 40℃, with a rotation speed of 100 rpm and the organic solvent was evaporated under reduced pressure in a rotary evaporator. Liposome suspension was obtained after all the organic solvent was removed from the mixture. The suspension was concentrated to reduce the size of liposomes with probe ultrasound. After determination of content, the suspension was freeze-dried with 1 mL liposome and 1 mL (50 mg/mL) sucrose solution in each vial.

One of the main problems of liposomal formulations is the stability. It is reported that the lyophilization technique represents a promising strategy to provide a stable formulation for a long period[23]. In the present study, sugars of 50 mg/mL was used as the protective agent to prevent liposome fusion and aggregation during the lyophilization. The freeze-drying process was carried out at -20 °C (±1 °C) at a pressure of 10^{-4} Torr during 12 h after the suspension was frozen at -80 °C overnight in glass vials. Water contents of liposomes obtained were less than 2 %, as determined by the Karl Fischer method[13]. Suspensions were reconstituted in 1 mL of pure water under vortex agitation. Lipo-HHT formulation as well as empty liposomes without DSPE-PEG2000 were prepared using the same procedures as those mentioned above.

Characterization

The morphological structure of HHT-liposomes was investigated using a transmission electron microscopy (TEM) (JEOL, JEM-2000EX, Japan) with negative staining by 2% phosphotungstic acid. The hydrodynamic diameter, size distribution and zeta potential of HHT-liposomes were examined using Zeta PALS instrument (Brookhaven Instrument Corporation) by dynamic light scattering (DLS) (Malvern Instruments). The liposome samples for zeta-potential analysis were diluted with phosphate buffered saline (PBS) (pH 7.4). Measurements were carried out at 25 ± 1°C.

In order to measure the amount of HHT encapsulated in the liposomes, the centrifugation method was used. Firstly, unencapsulated HHT was removed from the liposome suspensions, initially by centrifuging at 3000 rpm for 15 min in ultrafiltration centrifuge tube (10000 molecular weight cutoff), after which 2 mL of absolute methanol was added to the separated LCLipo-HHT to destroy and release the loaded HHT. After filtration, the amount of HHT encapsulated in the liposomes was measured by high performance liquid chromatography (HPLC) using a reverse-phase C18 column (250 mm×4.6 mm, 5 μm particle size; Shimadzu

Corp., Kyoto, Japan). Results shown in Figure S1 (Supporting Information) showed that the HHT peak was good with the retention time of 8.37 min. The mobile phase conditions were methanol-potassium dihydrogen phosphate solution (0.01 M) (KDP) (40:60, v/v) at 40 °C at a flow-rate of 0.8 mL/min[24]. The entrapment efficiency (EE) was calculated as equation (1):

$$EE\ (\%) = [HHT]_E/[HHT]_T \times 100\% \quad (1)$$

where $[HHT]_E$ was the amount of HHT entrapped in liposomes, and $[HHT]_T$ was the total amount of HHT added in the solution.

The drug loading capacity (LC) (mass %) was calculated as equation (2):

$$LC\ (\%) = W_E/W_P \times 100\% \quad (2)$$

where W_E was the amount of encapsulated drug, and W_P was the total amount of lipids in the formulation.

***In vitro* drug release**

To determine the kinetics of HHT release from liposomes *in vitro*, 1 mL of HHT-liposomes with 0.189 mg lipids were added to 1 mL fresh mouse plasma and the mixture was placed in dialysis bag (14000 molecular weight cutoff). The dialysis bag was immersed in 200 mL of pH 4.5 acetate buffer or pH 7.4 phosphate buffer and kept on water bath shaker at 37 °C. Samples (2 mL) were withdrawn and the same volume of fresh buffer medium was added periodically. 20 μL aliquots of the released drug were quantified at 288 nm by HPLC method as above mentioned. All measurements were taken in triplicate.

***In vivo* safety Evaluation**

The hemolytic potential of the HHT liposomes was determined and compared with that of the normal saline and purified water in rabbit blood[16]. Briefly, blood was withdrawn from New Zealand white rabbit and defibrinated by collection in vacutainer tubes. Erythrocytes were diluted by normal saline to 2% (v/v). The mixture of 2.5 mL of 2% erythrocyte solution and 2.5 mL of normal saline was used as control. New Zealand white rabbits (2 ± 0.05 kg) were provided by the Central Animal Laboratory of Southeast University. HHT injection (Inj-HHT), Lipo-HHT or LCLipo-HHT was injected intravenously into ear-edge of one of rabbit's ear once-a-day for 3 days. Normal saline was injected into the other ear as a control.

To investigate elementary toxicity of LCLipo-HHT, the mice were divided into four groups, and ten animals of each group. The mice were administered with normal saline,

Inj-HHT, Lipo-HHT or LCLipo-HHT, respectively, by intravenously (i.v.) injection into the tail vein on day 0 with a drug dose of 4 mg/kg. The systemic toxicity was assessed by measuring the body weight and the mortality of the mice for 14 days. Animals were euthanized and their heart, liver, and spleens were taken on day 14 after administration. The organs were fixed in 10% formalin, and blocks were paraffin embedded, sectioned, and stained with H&E using standard methods[25].

Pharmacokinetic and bio-distribution assay

The pharmacokinetic studies were carried out in BALB/c mice, weighing 20-25 g obtained from the Central Animal House of Southeast University. The mice were randomly divided into three groups, and 8 mice for each group. Lipo-HHT, LCLipo-HHT and HHT injection were injected intravenously into the tail vein of the mice (3 mg/kg). The blood samples were collected at predetermined intervals of 0.083, 0.25, 0.5, 1, 2, 4, 8, and 12 h post-dose into heparinized tubes.

Animals were sacrificed under ether anesthesia after blood samples drawn from orbit. Blood was collected in heparin-coated tubes and centrifuged immediately at 3500 rpm for 5 min to separate the plasma, and the plasma was stored at -20 °C until analysis. Heart, liver, and spleens were taken at 30 min, 4 h and 8 h, weighed and homogenized (10%, w/v) in a solution of 1% sodium bisulfate in normal saline. All samples were immediately frozen at -20 °C and analyzed in 3 days.

Biosamples (including plasma, heart, liver, and spleens) were collected as follows [26]. In brief, 10 μL harringtonine reference standard (80 ng/mL) and 200 μL dichloromethane was added to 100 μL aliquot of plasma or tissue homogenate (10%, w/v) with 1% sodium bisulfate. Then, the mixed samples were shaken and centrifuged at 12000 rpm for 5 min at 4 °C. A 20 μL aliquot of the supernatant fluid was injected into HPLC for assay. The equipment conditions were the same as *in vitro* drug release, and the mobile phase was composed of ammonium formate (0.1 M): methanol (55: 45). Elution was performed isocratically at 25 °C at a flow-rate of 0.8 mL/min[24].

All the pharmacokinetic parameters were calculated by the non-compartmental analysis using the DAS 3.0.5 software supplied by Tianjin University of Traditional Chinese Medicine (Tianjin, China).

Statistical analysis

The data obtained were expressed as mean ± SD (standard deviation). Statistical analysis was performed by Student's t-test for two groups, and one-way analysis of variance for multiple groups. A difference with $p < 0.05$ was considered as statistical significant. All experiments were conducted at least in triplicate.

Results and discussion

Preparation and characterization of HHT-loaded liposomes

In this study, liposomes with and without PEG 2000 were prepared using the ethanol injection and sonication method. During the process of liposome preparation, HHT should be homogeneously dispersed in phospholipid solution to ensure the HHT encapsulated in the lipid shell. The HHT-loaded liposomes were characterized by their size, zeta potential, encapsulation efficiency and drug loading capacity. The sizes of Lipo-HHT and LCLipo-HHT prepared by the ethanol injection method were 67.3 ± 2.9 nm and 75.6 ± 3.2 nm, respectively (Table 1). The PDI of liposomes prepared using both methods was reasonably low (<0.25). The zeta potentials of the Lipo-HHT and LCLipo-HHT were -21.3 ± 2.1 mV and -16.9 ± 2.5 mV, respectively. Besides, the zeta potential of empty liposomes was also tested as -25.3 ± 1.7 mV, which was a little lower than HHT-liposomes. The main reason could be speculated that when the hydrophobic drug HHT was efficiently encapsulated in the shell of liposomes, the nitrogen atom in the molecule of HHT may be protonated to influence the zeta potential. The encapsulation efficiencies were about 70% with LCs above 4.0%. The morphology of HHT liposomes was investigated by TEM (Figure 1). TEM results indicated that the liposomes were spherical in shape with negligible aggregation. The stability of the liposomes in freeze-drying was confirmed by the size measurement. Results show that the mean diameter of the LCLipo-HHT before and after lyophilization were 75.6 ± 3.2 nm and 79.1 ± 2.6 nm respectively, which demonstrated that there were no variations in the size of the liposomes before and after lyophilization.

***In vitro* drug release**

In vitro release of HHT from liposomes was investigated. As shown in Figure 2, after 24 h of dialysis in PBS (pH 4.5 and pH 7.4), the release rates of HHT from both drug loaded

liposomes were much slower than that of HHT injection control. The percentage of HHT released from HHT injection in the medium was more than 90.0% after 24 h. Both Lipo-HHT and LCLipo-HHT released HHT more than 50% in the first 2 hours. In the study, due to the alkaline characteristics of homoharringtonine (HHT) (pKa=12.9), at weak acid medium (pH 4.5), the speed of HHT released from HHT injection became much faster than Lipo-HHT and LCLipo-HHT at the initial hours (Figure 2A). However, at the pH 7.4 condition of Figure 2B, the speed of HHT released from HHT injection became a little slower than Lipo-HHT and LCLipo-HHT at the initial hours. The cumulative release of HHT were 57.8 and 62.9% for Lipo-HHT and LCLipo-HHT in pH 4.5 PBS solution, as well as 65.6 and 63.4% in pH 7.4 PBS solution, respectively. This could be explained that HHT was steadily incorporated into the lipid bilayer because of its similar solubility with cholesterol, and variation in lipid concentrations may also modify the drug release[27]. Nevertheless, as observed in Figure 2, there was no significant difference in drug release between the bare liposome and PEGylated liposome at different pH values (pH 4.5 and 7.4, respectively) ($p > 0.05$). The reason may be related to the fact that chain length of PEG (DSPE-PEG 2000) is not long enough to effect drug release. Therefore, the overall results showed that the liposomal formulations used in the study exhibited a sustained release behavior *in vitro*, which likely would lead to a prolonged action *in vivo*.

Safety Evaluation

For safety assay of LCLipo-HHT, hemolysis test *in vitro*, vascular irritation test in rabbits, and acute toxicity test *in vivo* were carried out. Hemolysis analysis was performed in order to estimate the HHT loaded liposomes hemocompatibility after intravenous administration. The results showed that there was no agglutinated erythrocytes observed under microscope after 0.0016-0.0064% Lipo-HHT, LCLipo-HHT, or HHT injection was added in 2% erythrocyte solution for 0.5-3 h. Similar values were obtained for the liposomes with no detectable rupture in the red blood cell membranes. In contrast, pure water, used as a control, had a complete hemolytic activity (100%) in the experiment. Therefore, these results indicate that LCLipo-HHT have an adequate hemocompatibility in the systematic administration at a dose of 0.016 mg/mL. The results of vascular irritation in rabbits were shown in Figure 3. Results showed that there was no thrombus, swelling, degeneration or

inflammatory cell infiltration observed after intravenous administration of LCLipo-HHT at the dosage of 0.1 mg/kg in the pathology slide photomicrographs of normal saline, Lipo-HHT, LCLipo-HHT, HHT injection groups.

Moreover, different doses were added in the experiment to ensure the objectivity of the evaluation. Dosage of 2 mg/kg, 4 mg/kg, 6 mg/kg were given to mice for toxicity evaluation, respectively. The results showed that there were no discrimination of survival for 2 mg/kg group, and all the mice died within 48 hours for 6 mg/kg dosage injection group. Thus, dose of 4 mg/kg was chosen for toxicity evaluation (as shown in Table S1 and S2 in Supporting Information). The percentage of survival rates was shown in Figure 4A and the change of acute toxicity in body weight was also measured for each survival mice as shown in Figure 4B. With the 4 mg/kg dose for injection, the mice of normal saline group showed an increase in body weight without any observable side effects. From experimental results, mice in the Lipo-HHT, LCLipo-HHT and HHT injection treated group induced a significant influence in body weight compared with normal saline group ($p < 0.01$). Besides, weight gain of LCLipo-HHT group was much faster than Lipo-HHT and HHT injection group ($p < 0.05$), which may because that the LCLipo-HHT acted with less drug toxicity.

Compared to the dose of 2.0 mg/kg and 6.0 mg/kg shown in Table S1 and Table S2, the survival rates were 100%, 50%, 60%, and 70% when mice received normal saline, HHT injection, Lipo-HHT, and LCLipo-HHT at a dose of 4.0 mg/kg after drug administration for 4 days, respectively. From the results, it can be found that the survival rates of LCLipo-HHT were higher than that of Lipo-HHT and HHT injection at the same dose. The main reason could be speculated as the LCLipo-HHT released their drug content at a much slowly rate, which decreased the systemic toxicity[25].

Histological analysis was used to confirm the cardiotoxicity, hepatotoxicity and splenic toxicity of LCLipo-HHT, as shown in Figure 5. The results of heart slice showed no significant difference among normal saline group, HHT injection group, Lipo-HHT group, and LCLipo-HHT group, no degeneration and necrosis was observed. The results suggested there was no obvious heart toxicity observed in LCLipo-HHT group in acute toxicity test. Moreover, there was no degeneration and necrosis observed in liver and spleen pathology slice. In summary, it could be concluded that the LCLipo-HHT could not cause further

damage of the liver, spleen and heart compared to control group.

Pharmacokinetic and bio-distribution assay

In order to investigate the pharmacokinetic and bio-distribution, the HHT injection, Lipo-HHT and LCLipo-HHT were administered intravenously with a dose of 3 mg/kg in mice. The HHT concentration in the blood was determined by HPLC as the above-mentioned parameters. The results showed that HHT injection had a short circulation half-life (elimination phase $t_{1/2(\beta)}$ of 3.23 h) and rapid elimination rate (Cls = 27.16 L/(kg·h)) from the plasma (Figure 6). PEGylated liposomal encapsulation of HHT caused obvious changes in pharmacokinetic parameters: the LCLipo-HHT were more slowly removed compared with HHT injection. The elimination phase ($t_{1/2(\beta)}$ = 4.47 h) and mean residence time (MRT = 5.35 h) of LCLipo-HHT were 1.38 and 1.34 times longer than those of HHT injection, respectively. Besides, the area under curve (AUC) of LCLipo-HHT (198.27 μg/L) was 1.47 and 1.99 times higher than Lipo-HHT and HHT injection, respectively. Overall, the PEGylated liposomes had comparatively better pharmacokinetic profiles than the bare liposomes and the drug solution.

The HHT distribution in different organs were shown in Figure 7. From the results, it was clear that HHT was widely and rapidly distributed into all the assessed organs following intravenous administration of Lipo-HHT, LCLipo-HHT and HHT injection. After administration for 30 min (Figure 7 A), HHT was mainly distributed over the kidney, spleen and liver, followed by the heart and lung. HHT injection (3.16 μg/100mg) had a much higher ratio than Lipo-HHT and LCLipo-HHT in kidney. LCLipo-HHT (2.25 μg/100mg) retained the highest HHT distribution in spleen compared with other formulations 4 hours after administration (Figure 7 B). The tissue distribution of 8 hours after administration was shown in Figure 7 C, it can be found that the concentrations of HHT for Lipo-HHT and LCLipo-HHT in spleen were 1.48 and 1.66 μg/100mg, which were much higher than other tissues. In this study, tissue distribution of HHT injection in all tissues had the highest concentration at 30 min, as the first time point of detection. However, the proportion of HHT in spleen and liver was much lower than that of other organs, which may be due to the drug accumulation in these tissues[28]. Additionally, the results showed that HHT in liposomes had a much lower concentration than HHT injection, especially for LCLipo-HHT. The

bio-distribution results here demonstrated that HHT liposomes can potentially reduce the cardiotoxicity.

Conclusion

In summary, we have developed PEGylated liposomes to encapsulate homoharringtonine-a potent antileukemic drug. The PEGylated liposomes showed high EEs and LC, with size around 70 nm and PDI below 0.25, which are favorable for pharmaceutical applications of nanomedicines. Specifically, incorporation of PEG-derivatives into HHT PEGylated liposomes decreased uptake efficiency by RES resulting in a prolonged circulation time and enhanced safety *in vivo*. However, accumulation of PEGylated liposomes in some organs like liver and spleen should also be concerned in future study, which may cause the potential long-term toxicity. Overall, the PEGylated liposome formulation of HHT could probably provide a good alternative with sustained delivery properties as well as better safety for future effective treatment of leukemia in the clinic.

Acknowledgments

This work was supported by the National Important Science Research Program of China (Nos. 2013CB733804), National Natural Science Foundation of China for Key Project of International Cooperation (61420106012), National Natural Science Foundation of China (31370019, 81301870, 81473160), Natural Science Foundation of Anhui Province (1508085MH203), and Collaborative Innovation Center of Suzhou Nano Science and Technology as well as Collaborative Innovation Center of Dendrobium Industrialization of Anhui Province.

Competing Interests

The authors have declared that no competing interest exists.

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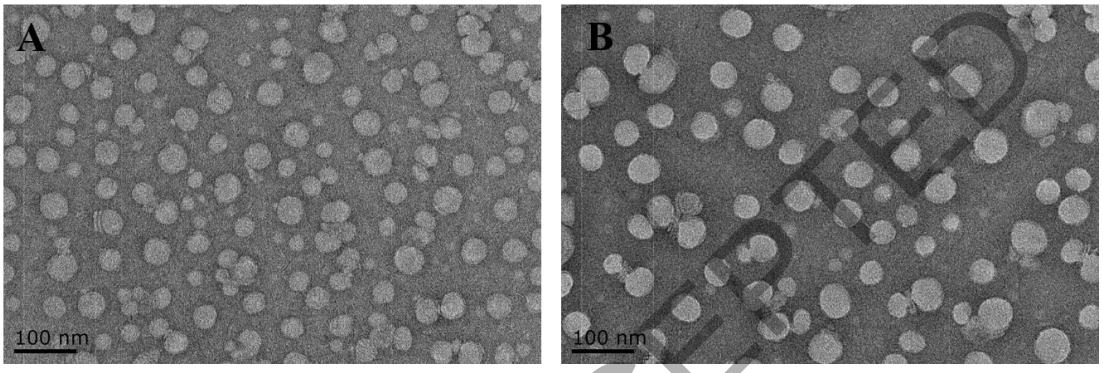


Figure 1. TEM images of reconstituted from lyophilization powder of Lipo-HHT (A) and LCLipo-HHT (B). Liposomes were negatively stained with phoshotungstic acid. Scale bar represents 100 nm.

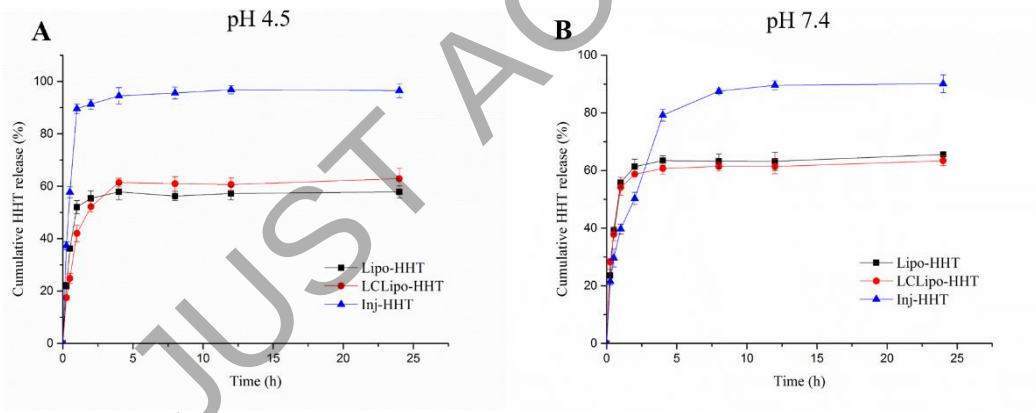


Figure 2. Graph showed the release kinetics of HHT from Lipo-HHT and LCLipo-HHT in 24 h at 37 ^{o}C (pH=4.5 (A), pH=7.4 (B)). Data represent mean ± SD, n=3.

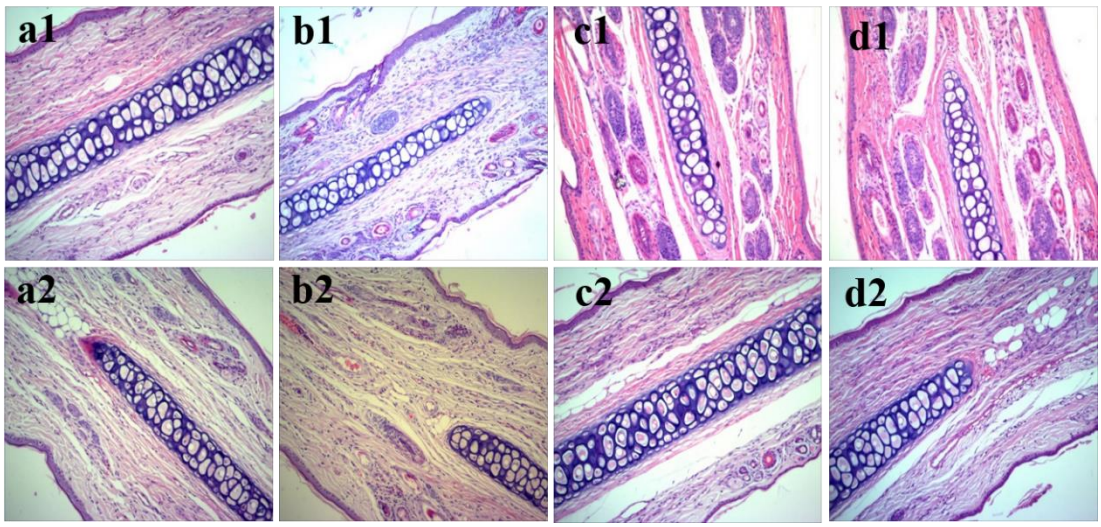
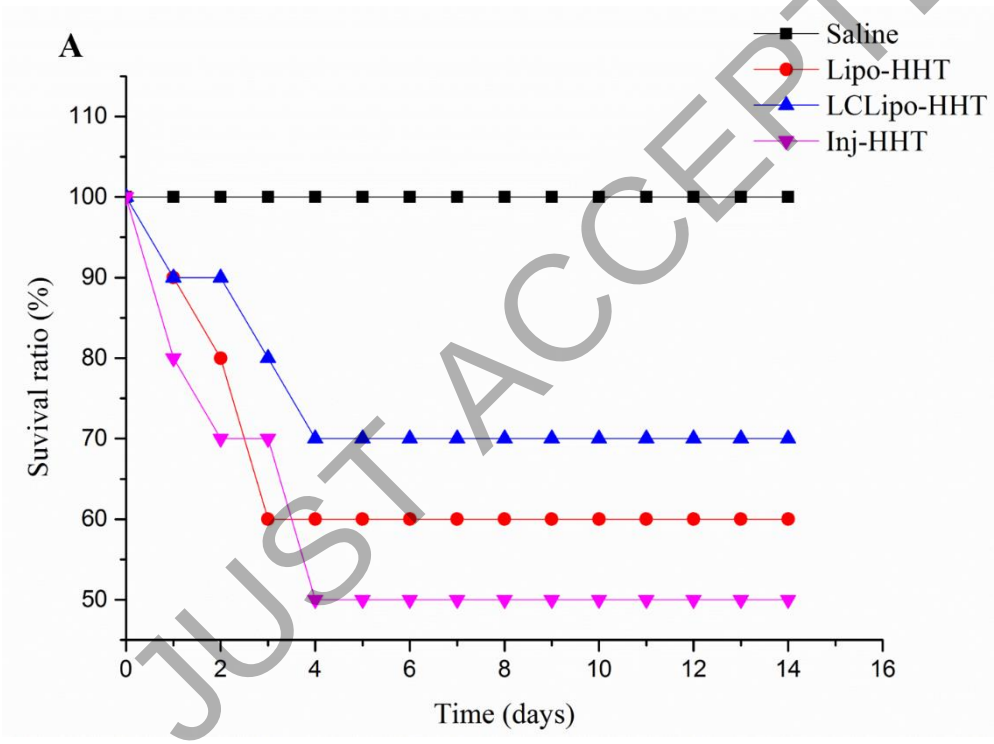


Figure 3. Representative pathology slide photomicrographs of rabbit ear vein slices in vascular irritation test. a) Normal saline (control); b) Lipo-HHT; c) LCLipo-HHT; d) Inj-HHT. 1: near to injection site (1.3 cm); 2: far from injection site (4.0 cm).



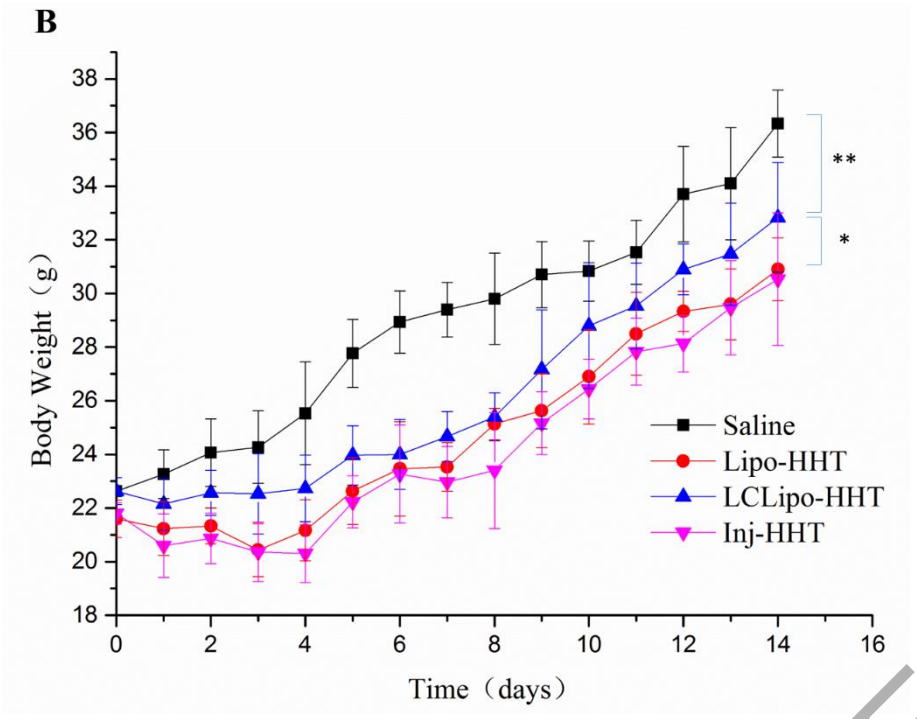


Figure 4. Acute toxicity test studies of Lipo-HHT, LCLipo-HHT and Inj-HHT in normal mice at a dose of 4 mg/kg. (A) Survival rates of mice treated with normal saline, Lipo-HHT, LCLipo-HHT and Inj-HHT. (B) Changes in relative body weight of the mice treated with normal saline, Lipo-HHT, LCLipo-HHT and Inj-HHT. Data are presented as the mean ± SD, $n \geqslant 5$. (*$p < 0.05$, **$p < 0.01$).

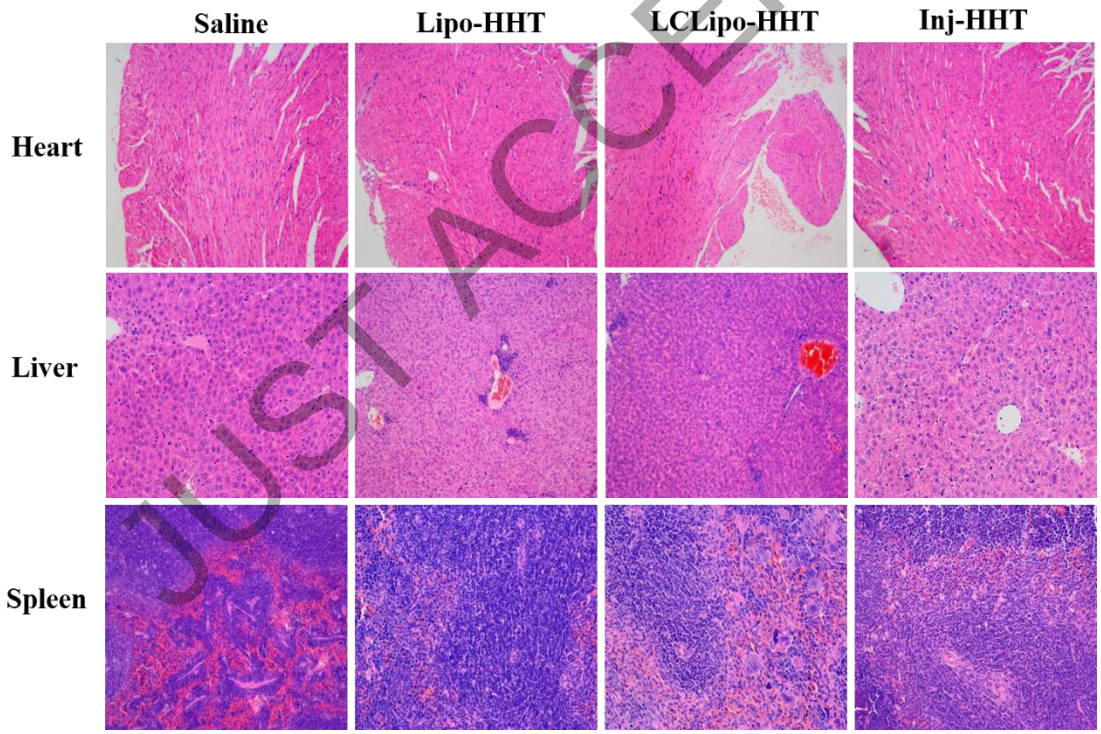


Figure 5. Representative histological H&E staining of heart, liver and spleen tissues obtained from healthy mice at day 14 after intravenous injection of normal saline, Lipo-HHT, LCLipo-HHT and Inj-HHT at equivalent dose of 4.0 mg/kg (magnification 400×).

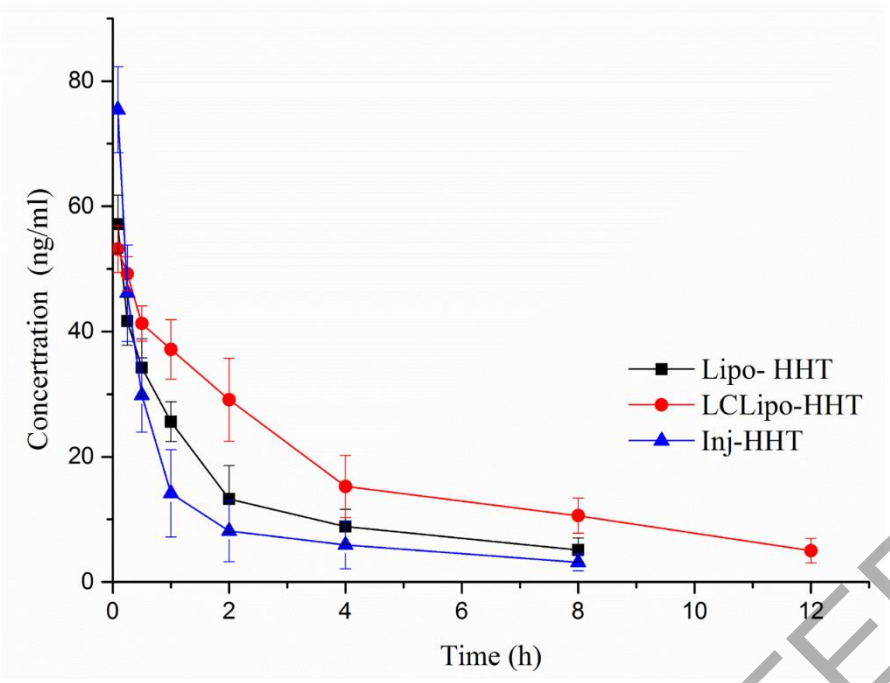
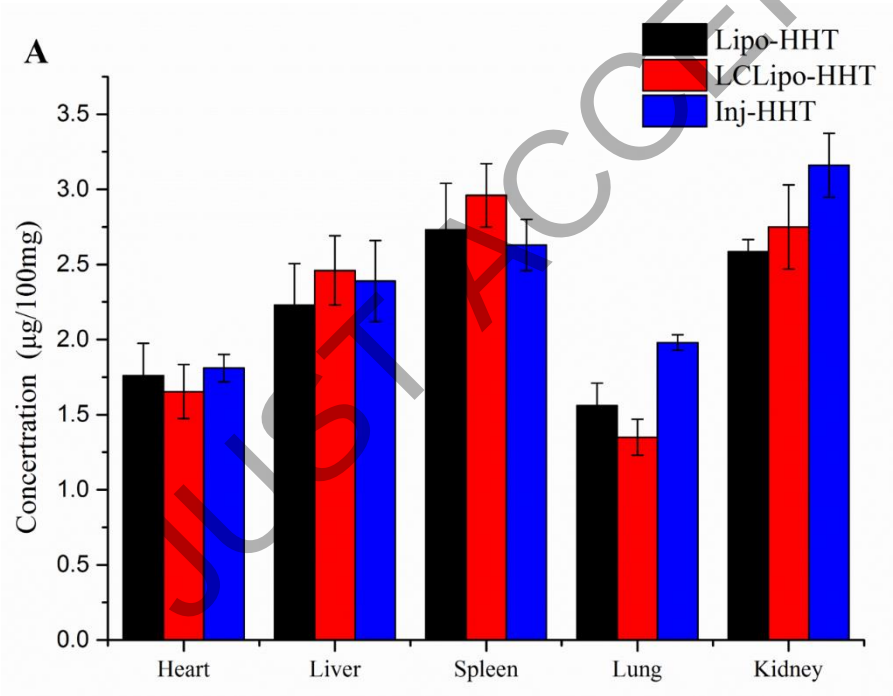


Figure 6. The concentration-time curves of HHT in plasma of mice given Lipo-HHT, LCLipo-HHT and HHT Injection after intravenous administration. Each point represents the mean ± SD of three mice.



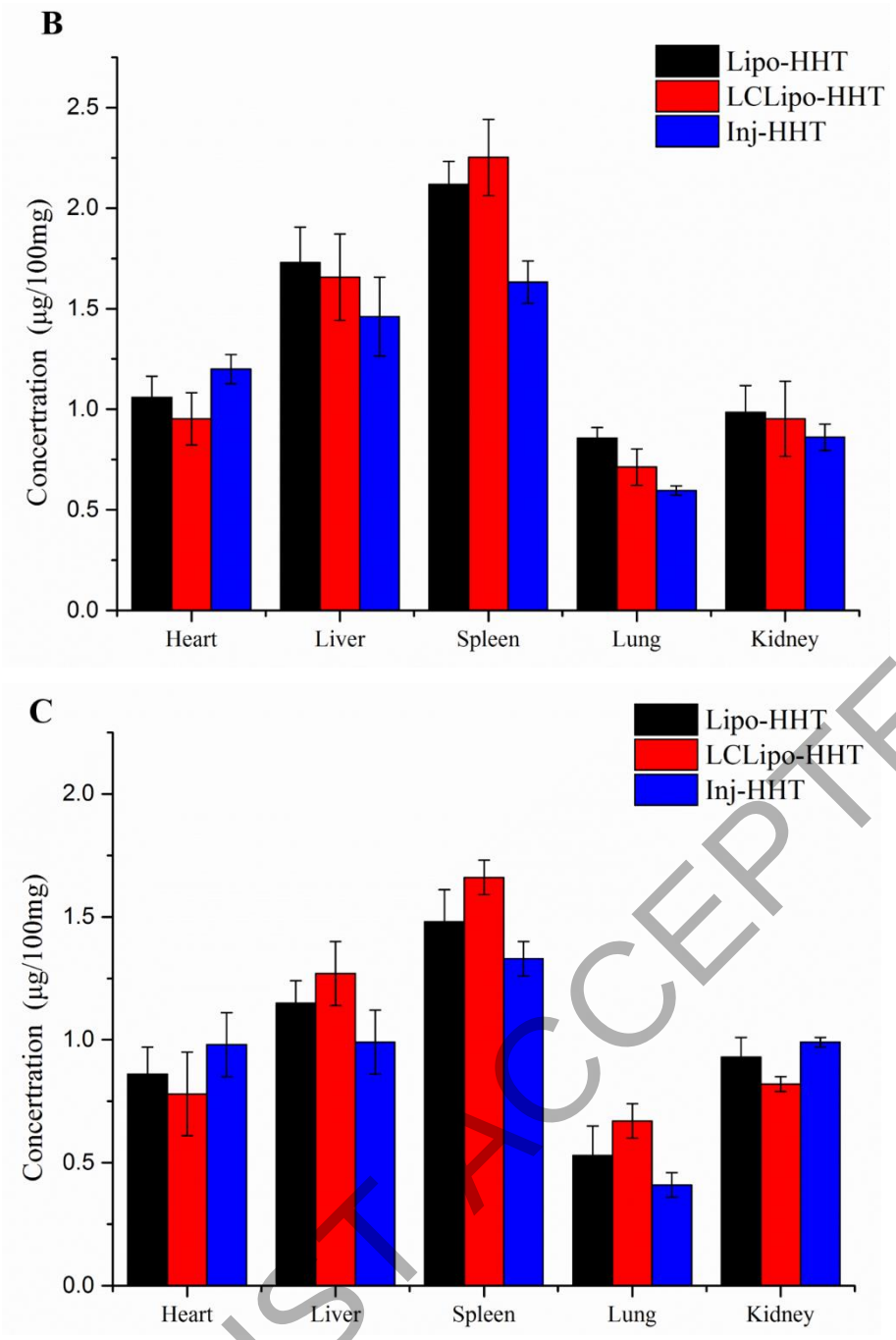


Figure 7. Bio-distribution of Lipo-HHT, LCLipo-HHT and HHT injection in BALB/c mice following a single injection after (A) 30 min, (B) 4 h, (C) 8 h.

Table 1. Physicochemical characteristics of the HHT-loaded liposomes[a].

Liposomes	Size(nm)	PDI	Zeta potential (mV)	EE (%)	LC (%)
Lipo-HHT	67.3 ± 2.9	0.23	-21.3 ± 2.1	71.3 ± 2.6	4.1 ± 0.3
LCLipo-HHT	75.6 ± 3.2	0.25	-16.9 ± 2.5	69.5 ± 1.7	4.3 ± 0.5

[a] PDI: polydispersity index, Values are given as mean ± SD (n = 3).