Isolation of herpetin from *Herpetospermum* seed and hepatoprotective activity of liposomal herpetin against carbon tetrachloride-induced liver injury in mice

Jian Gu1, Zhixiang Yuan2, Rui Tan3, Xin Zhang1

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Rui Tan, College of Medicine, Southwest Jiaotong University, Chengdu 610031, China
tanrui@swjtu.edu.cn


Objective: The aims of this study were to demonstrate the hepatoprotective activity of herpetin (HPT) and the enhanced hepatoprotective efficiency of liposomal herpetin against carbon tetrachloride-induced liver injury in mice.

Methods: Herpetin was isolated from Herpetospermum seed and identified by ESI-MS and NMR. To enhance liver targeting and improve solubility of HPT, liposomal HPT was prepared with optimal formulation. The intravenous injection safety of the liposomes was then evaluated. Further, the hepatoprotective effects of liposomal HPT on model mice were investigated by the comparison of different liver marker enzymes and histopathological examination.

Results: The prepared HPT liposome showed spherical or ellipsoidal vesicles with the entrapment efficiency of 94.50 ± 2.15% and particle size of 119.2 ± 10.7 nm. After 4 days intravenous administration of liposomal herpetin, no obvious damage could be observed at the injection site of each group. The liposomal HPT has no destructive effect on erythrocytes and little influence on whole blood clotting time. Free HPT exhibited only a weak protective function to model mice, whereas an enhanced hepatoprotective activity was observed using liposomal herpetin for treatment.

Conclusion: The hepatoprotective efficiency of herpetin is able to be promoted through pharmaceutical application of liposome and liposomal herpetin is a promising new medicine for hepatoprotection.

1. Introduction

Hepatitis is one of the most common liver diseases and characterized by the presence of inflammatory cells in the organ. It is mostly caused by immune cells in the body attacking the liver and causing autoimmune hepatitis under the condition of infections, hepatotoxic drugs, alcohol, etc. Hepatic inflammation is responsible for liver cells damage, fibrosis, and cirrhosis. Although there are many chemical drugs for treating these diseases, limited efficacy is obtained (Zhang et al. 2012). Therefore, the increasing demands for low toxicity drugs with good therapeutic performance are appealing to find new compounds in plants. Numerous positive results on significant hepatoprotective activity of plant drugs have been reported, which has gained momentum over the years (Li et al. 2013; Yin et al. 2014; Yu et al. 2014).

*Herpetospermum* seed, a common medication used for liver diseases as a folk medicine, is the dried ripe seed of *Herpetospermum caudigerum* Wall. It is distributed in south-west China, Nepal, and northeast India, at an altitude of 2300-3500 m. Ethanolic extracts of *Herpetospermum* seeds have been reported to have hepatoprotective potential. *Herpetospermum* seeds contain a large amount of lignans, such as herpetal, herpetriol, herpetrione and so on, which showed anti-liver injury and anti-hepatitis effects (Yuan et al. 2005). Herpetin (HPT) is a new bioactive lignan isolated firstly from *Herpetospermum* seed in 2005 (Fig. 1) and it exhibits it’s effects in reducing the replication and expression of HbsAg and HbeAg, and has significant inhibitory effect on HBV-DNA in vitro (Yuan et al. 2005). However, it is unknown whether herpetin is protecting against liver injury. Therefore, in the present work an attempt has been made to study the anti-liver injury function of HPT in CCl4 induced-liver mice. Firstly, HPT was isolated and purified according to Yuan’s method with some improvements (Yuan et al. 2005). The
structure of HPT was elucidated by means of ESI-MS and NMR. Unfortunately, HPT is a poorly soluble drug with a solubility of less than 0.1 mg/mL in water and exhibited weak hepatoprotective efficiency. The similar negative results were reported by Tasadq et al. (2003). Increasing the liver targeting profile of HPT may allow more effective incorporation for treatment. To achieve the therapeutic purpose, liposome as a strategy for pharmaceutical modification is adopted to improve the solubility and enhance the liver targeting profile of HPT.

With this consideration in mind, liposomal HPT was prepared with optimal formulation design. Then, lyophilization of liposomal HPT was adopted for long-term storage. Meanwhile, the intravenous injection safety of liposomal HPT with perfect entrapment efficiency was evaluated. Finally, the model mice of CCl4-induced liver injury were employed with pre-treatment of free HPT or liposomal HPT. The Liver marker enzymes, such as serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and malondialdehyde (MDA) and superoxide dismutase (SOD) were measured and compared. In addition, histopathological examination of the livers was also performed. The aims of the present study were to assess whether HPT or liposomal HPT possesses in vivo hepatoprotective effects against CCl4-induced liver injury in mice, explore the possible mechanism and lay the foundation for a promising new drug for hepatoprotection.

2. Investigations and results

2.1. Isolation and identification of HPT

Hepatoma was isolated according to the procedure described by Yuan et al. (2005). The structure was identified by MS, 1H NMR, 13C NMR.

2.2. Preparation of liposomal HPT

Three most common methods available for liposome preparation, film dispersion method, injection method and anti-phase evaporation method were applied in the experiments. As shown in Table 1, the film dispersion method is the best way to prepare HPT liposomes with small particle size, high stability and EE. To maximize the entrapment efficiency, an uniform design of mixture experiments with constraints was further applied to optimize the formulation. The factors, levels and experimental results are shown in Table 2. Using the soft analysis function, a polynomial equation was fitted as follows: \( y = 67.2 - 16.5x_1 + 184.96x_2 + 506.25x_3 - 436.81x_2x_3 - 2784.6x_1x_2x_3 \). The equation was evaluated for statistical significance (\( p < 0.05 \)). Thus, the optimal formulation could be predicted, namely, 78.45% phospholipids, 19.51% cholesterol and 2.15% HPT. In addition, the concentration of F68 and sonic time were also evaluated by single-factor test. The data showed that EE of HPT liposome were 78.45 \pm 2.51\%.

Table 1: Effects of different preparation methods on the properties of liposomal HPT

<table>
<thead>
<tr>
<th>Method</th>
<th>Visual appearance</th>
<th>Particle size (( \mu m ))</th>
<th>EE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Film dispersion method</td>
<td>No aggregation</td>
<td>113.7 \pm 19.8</td>
<td>90.78 \pm 2.51</td>
</tr>
<tr>
<td>Injection method</td>
<td>Aggregation within 24 h</td>
<td>107.6 \pm 20.3</td>
<td>40.62 \pm 6.18</td>
</tr>
<tr>
<td>Anti-phase evaporation method</td>
<td>No obvious aggregation</td>
<td>386.7 \pm 36.7</td>
<td>84.65 \pm 5.40</td>
</tr>
</tbody>
</table>

Data were expressed as the mean \( \pm \) SD (n = 3)

Table 2: Uniform design

<table>
<thead>
<tr>
<th>No.</th>
<th>( x_1 ) (%)</th>
<th>( x_2 ) (%)</th>
<th>( x_3 ) (%)</th>
<th>EE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>84.9</td>
<td>12.3</td>
<td>2.71</td>
<td>88.3</td>
</tr>
<tr>
<td>2</td>
<td>80.8</td>
<td>12.9</td>
<td>6.28</td>
<td>80.3</td>
</tr>
<tr>
<td>3</td>
<td>79.1</td>
<td>19</td>
<td>1.92</td>
<td>90.5</td>
</tr>
<tr>
<td>4</td>
<td>77.5</td>
<td>11.8</td>
<td>10.7</td>
<td>76.8</td>
</tr>
<tr>
<td>5</td>
<td>75.9</td>
<td>18.3</td>
<td>5.76</td>
<td>82.3</td>
</tr>
<tr>
<td>6</td>
<td>73.2</td>
<td>16.5</td>
<td>10.4</td>
<td>72.4</td>
</tr>
<tr>
<td>7</td>
<td>70.7</td>
<td>13.7</td>
<td>15.7</td>
<td>62.3</td>
</tr>
<tr>
<td>8</td>
<td>67.3</td>
<td>18.2</td>
<td>14.6</td>
<td>50.1</td>
</tr>
<tr>
<td>9</td>
<td>62.3</td>
<td>18.7</td>
<td>19</td>
<td>42.5</td>
</tr>
</tbody>
</table>

* According to the rule of uniform design for constrained mixture experiment, the main ingredients should obey the equation: \( y = 67.2 - 16.5x_1 + 184.96x_2 + 506.25x_3 - 436.81x_2x_3 - 2784.6x_1x_2x_3 \). The pre-experimental results suggested the concentration range should be set at 60\% \( \leq x_1 \leq 80\% \), 10\% \( \leq x_2 \leq 30\% \) and 15\% \( \leq x_3 \leq 30\% \). The polynomial equation \( y = a_0 + a_1x_1 + a_2x_2 + a_3x_3 + a_12x_1x_2 + a_13x_1x_3 + a_23x_2x_3 + a_123x_1x_2x_3 \). The pre-experimental results suggested the equation range should be set at 60\% \( \leq x_1 \leq 80\% \), 10\% \( \leq x_2 \leq 30\% \) and 15\% \( \leq x_3 \leq 30\% \).

0.5% F68 is enough to approach satisfactory results. With the increase of sonic time from 5 min to 20 min, EE of liposome rose from 82.65 \pm 2.14\% to 93.29 \pm 3.26\%. With further prolonged sonication treatment, no significant increase of EE was observed. Therefore, 0.5% F68 was finally adopted for liposome preparation with sonication treatment for 20 min.

According to the results mentioned above, the optimized preparation could be defined. In brief, 24.4 mg HPT, 780.5 mg phospholipids and 195.1 mg cholesterol were dissolved in chloroform in a round-bottom flask. The lipid film was formed by eliminating the organic solvent under reduced pressure. Then, the thin film was swollen in 50 ml of 0.5% F68 solution for 30 min and sonicated in a water bath sonicator for 20 min. The obtained liposomes were filtered through a 0.22 \( \mu m \) filter. The prepared HPT liposomes showed spherical or ellipsoidal shape with good dispersity under TEM (Fig. 2A). The EE and particle size were 94.50 \pm 2.15\% and 119.2 \pm 10.7 nm, respectively, with a PDI of 0.156 \pm 0.075 (Fig. 2B). The release profile of liposomal HPT is presented in Fig. 2C. A fast release of HPT during the initial stage and consecutively a sustained release in the following stage were observed. The release data were modeled using Higuchi, first-order and zero-order model (Table 3), which indi-
Fig. 3: The irritation test by intravenous administration. Histopathological examination of rabbit ears: A: saline group; B: HPT liposome group. Magnification: ×100.

Table 3: Fitting of release model of HPT liposomes in vitro

<table>
<thead>
<tr>
<th>Model</th>
<th>Fitting equation</th>
<th>Correlation coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Higuchi</td>
<td>$y = 0.125x^{1/2} + 0.107$</td>
<td>$r^2 = 0.8494$</td>
</tr>
<tr>
<td>first class</td>
<td>$y = 0.948(1-e^{-0.132x})$</td>
<td>$r^2 = 0.9945$</td>
</tr>
<tr>
<td>zero class</td>
<td>$y = 0.012x + 0.313$</td>
<td>$r^2 = 0.664$</td>
</tr>
</tbody>
</table>

cated that the release profile reasonably well fits the first-order model.

2.3. Optimal lyophilization of HPT liposome

Glucose, mannitol, lactose and sucrose as the common lyoprotectants were used alone or in combination for optimal lyophilization. As shown in Table 4, generally, lyoprotectants with lower concentration could cause higher HPT leakage, while lyoprotectants containing mannitol might remarkably enlarge the particle size in spite of good appearance obtained. The process of freeze-drying had no effect the particle size of rehydrated liposomes containing 5% lactose combined with 5% sucrose and did not cause any drug leakage, which indicated that this combination of lyoprotectants was much suitable for the preparation of HPT liposome lyophilization. The residual water content (w/w) in the samples of the optimal lyophilization liposome was $1.27 \pm 0.12\%$ ($n = 3$), which met the regulatory requirement.

2.4. Safety of liposomal HPT for i.v. injection

After 4 days intravenous administration, no obvious damage could be observed at the injection site of each group. There was no thrombus, angiectasia or vascular congestion in blood vessel at or beside the injection site under histopathologic examination (Fig. 3A-B). In addition, tissues surrounding the injection site were normal without any signs of dropy, hemorage, inflammatory cell infiltrate or endothelial cell necrosis and degeneration. The results indicated that intravenous injection of liposomal HPT did not cause vascular irritation. As summarized in Fig. 4A, the hemolysis rates of 2%, 4% and 6% liposome were lower than the safe value of 5% according to ISO 10993-4:2002 (Zhang et al. 2009), suggesting that the liposomal HPT has no destructive effect on erythrocytes. The curves of whole blood clotting time of liposomes and physiological saline is drawn in Fig. 4B. It is obvious that there was no difference between liposome and saline in clotting time. Liposomal HPT showed little influence on whole blood clotting time. From the results mentioned above, we preliminarily ensured liposomal HPT is safe for intravenous injection.

2.5. Hepatoprotective activity of liposomal HPT

Carbon tetrachloride-induced acute liver injury can be identified by elevated serum ALT, AST and ALP levels in mice. As shown in Fig. 5 (A-C), the levels of serum ALT, AST and ALP in the normal group were very low, while those in the control group were dramatically higher due to the CCl4 injury. However, pretreatment with free HPT, Qing Kai Ling injection and liposomal HPT in other groups for 7 consecutive days reduced the elevation of serum ALT, AST and ALP levels. Of the three groups, the HPT group showed a decrease trend, however, no obvious difference was observed with the control group ($p > 0.05$). Interestingly, the activities of serum ALT, AST and ALP in the liposomal HPT group were significantly lower than those in the HPT group ($p < 0.05$).

Histopathological observation of liver section for the normal group exhibited a normal cellular architecture with regular arrangement of hepatic cells, sinusoidal spaces and a central vein (Fig. 6A). In comparison, the control group showed severe degenerative changes and the hepatic cells presented fatty...
Table 4: Optimized preparation of HPT lyophilized liposomes

<table>
<thead>
<tr>
<th>Lipoprotectants</th>
<th>Appearance</th>
<th>Dispersion</th>
<th>Particle size (nm)</th>
<th>EE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5% Glucose</td>
<td>bad</td>
<td>good</td>
<td>898.7 ± 9.1</td>
<td>93.37 ± 0.19</td>
</tr>
<tr>
<td>5% Mannitol</td>
<td>bad</td>
<td>good</td>
<td>2694 ± 25</td>
<td>97.25 ± 0.45</td>
</tr>
<tr>
<td>2.5% Glucose + 2.5% Mannitol</td>
<td>bad</td>
<td>good</td>
<td>1591 ± 16</td>
<td>91.17 ± 0.46</td>
</tr>
<tr>
<td>5% Lactose + 5% Sucrose</td>
<td>good</td>
<td>good</td>
<td>107.0 ± 1.2</td>
<td>99.70 ± 0.50</td>
</tr>
<tr>
<td>10% Sucrose</td>
<td>average</td>
<td>good</td>
<td>253.1 ± 0.9</td>
<td>67.10 ± 0.62</td>
</tr>
<tr>
<td>3% Glucose</td>
<td>good</td>
<td>good</td>
<td>493.8 ± 5.1</td>
<td>70.10 ± 0.35</td>
</tr>
<tr>
<td>3% Mannitol</td>
<td>excellent</td>
<td>good</td>
<td>493.0 ± 59</td>
<td>58.61 ± 0.93</td>
</tr>
<tr>
<td>1.5% Glucose + 1.5% Mannitol</td>
<td>good</td>
<td>good</td>
<td>253.0 ± 21</td>
<td>64.65 ± 0.21</td>
</tr>
<tr>
<td>1% Glucose + 1% Sucrose + 1% Lactose</td>
<td>good</td>
<td>good</td>
<td>119.6 ± 2.1</td>
<td>65.19 ± 0.38</td>
</tr>
</tbody>
</table>

Data were expressed as the mean ± SD (n = 3).

Degeneration, necrosis and inflammation (Fig. 6B). After the 7 consecutive administrations in advance, a certain alleviation of pathological changes could be observed in the HPT group (Fig. 6C). In addition, a dramatic reduction of degeneration, necrosis and inflammation was achieved in the positive group and the liposomal HPT group (Fig. 6D-E). A further evaluation of MDA and SOD is shown in Fig. 6F. The production of MDA increased and SOD decreased remarkably in the control group, when compared with the normal group. Similarly, certain improvements were also observed in the HPT and the positive group with the comparison to the control group. Obviously, liver marker enzymes in the liposomal HPT group were improved sharply and its function for MDA and SOD improvement was more efficient than in the HPT group (p < 0.05). A similar trend can be seen, being consistent with the histopathological observation. The findings therefore supported the marker enzymes data in advocating the superior efficacy of HPT when it was loaded to liposomes.

3. Discussion

Yuan et al. first isolated HPT from Herpetospermum caudigerum using semi-preparative HPLC and proved inhibitory effects of HPT on HBV-DNA and the replication and expression of HBsAg and HBeAg (Yuan et al. 2006, 2005). In this paper, HPT was also prepared using a Sephadex LH-20 column. Data of MS, 1H NMR and 13C NMR spectra were used to identify the structure of obtained HPT, which were consistent with Yuan’s report.
To investigate whether herpetin improves resistance to liver injury, three doses of HPT (1 mg/kg, 10 mg/kg and 20 mg/kg) were given to mice prior to CCl4 injection in our preliminary experiment. However, even at the high dose of 20 mg/kg, HPT still exhibited a weak protective function to the model mice. Additionally, owing to the poor solubility of HPT (less than 0.1 mg/mL in water), we found it difficult to solubilize HPT using surfactant or organic solvents to safely increase treatment dose. In order to solve the problem, liposomes as carriers came under our consideration. Liposomes are accepted as potent carriers not only for their biocompatible nature but also for increasing the dissolution of poorly soluble drugs. Furthermore, because of the presence of efficient capture by liver, liposomes are effective in the site-specific drug delivery to hepatic tissue. It is reported that liposomes are largely taken up by Kupffer cells whose high endocytic activity makes them most competent to internalize colloidal particles like liposomes (Mandal et al. 2007).

With the constrained mixture design, the optimal ratio of main ingredients in the liposome formulation was determined. Mixture designs are common in pharmaceutical applications for natural reasons and are applied both for screening and optimization studies (Gabrielsson et al. 2002; Shen et al. 2014; Tan et al. 2011). Compared with other design methods, the uniform design cannot only greatly reduce the test points but also obtain a better result because that test point is more evenly distributed in the test range (Cao et al. 2013). It is very suitable for resolving the formulation problem with multiple factors and levels. In this study, the entrapment efficiency achieved for optimal liposomes using uniform design was found to be 94.50 ± 2.15% with excellent homogeneity and sustained release.

Due to physical and chemical instabilities (e.g. hydrolysis and oxidation of HPT and phospholipids, HPT leakage and liposomes aggregation) of liposomal HPT in aqueous dispersions, lyophilization should be used extend the shelf-life of HPT liposomes. The protective effect during liposome lyophilization is...
Lipid peroxidation is a major cause of CCl₄ mediated liver hepatocyte degeneration and necrosis and decreased inflammation of the liver. Also showed the advance of liposomal HPT in alleviating the specificity of liposomal HPT. In parallel with the alteration of the indexes. This is probably attributed to the intrinsic liver target hepatoprotective effects, which was consistent with previous reports. In our study, administration of CCl₄ induced liver injury in ICR mice with obvious increase in ALT, AST and ALP level as well as the protection of liver tissue. In conclusion, liposomal HPT exhibited more hepatoprotective efficiency in model mice, than free HPT including the decrease of ALT, AST and ALP level in circulation, the improvement of MDA and SOD indexes in liver and the protection of liver histological structure. Thus, the hepatoprotective efficiency of herpetin can be promoted through pharmaceutical application of liposomes and liposomal herpetin is a promising new medicine for hepatoprotection.

4. Experimental

4.1. Chemicals

Phospholipid was supplied by Lipoid Company (Ludwigshafen, Germany). Chelex-100, silica gel and Sephadex LH-20 were obtained from Sigma-Aldrich Company Ltd. (Gillingham, UK). Qing-Kai-Ling injection was manufactured by Guangzhou Mingxing Pharmaceuticals (Guangzhou, China). AST, ALT and ALP kits were purchased from Changchun Hu Li Biotech-nology Company Ltd. (Changchun, China). The commercial MDA and SOD kits were from Jiangxi Institute of Biotechnology (Nanjing, China). All other chemical reagents and solvents used were analytical grade.

4.2. Animals

ICR mice with an initial body weight 18–22 g were obtained from the Da-Shao Experimental Animal Ltd. (Beijing, China). These animals were allowed to acclimatize in environmentally controlled quarters (24 ± 1 °C and 12h light/dark cycle). Unless specified otherwise, water and food were given ad libitum throughout the experiment at libitum. All procedures of the animal studies were approved by Animal Care and Use Committee of Southwest University for Nationalities, and were performed according to the requirements of the People's Republic of China National Act on the use of experimental animals.

4.3. Separation and purification of HPT

Separation and purification of HPT was carried out according to Yuan et al. (2005). Dried powder of Herpetospermum caudigerum seeds (1.0kg) was extracted twice with 8L 80% ethanol solution for 2h followed by evaporation of ethanol at 70 °C. The extract was then partitioned into petroleum ether, ethyl acetate and n-butanol parts, respectively. The ethyl acetate fraction was concentrated and separated into 18 fractions (F1-18) with silica gel column chromatography using a methanol-chloroform gradient system. Of fractions, F1-10 was further isolated by bioassay using HPT by silica gel col-

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umn chromatography eluted with a petroleum:chloroform:acetic acid (1:1:1) system. After that, the crude HPT was purified on a Sephadex LH-20 column with methanol to yield HPT compound identified using NMR (Bruker Avance III 400 MHz spectrometer) and MS (Bruker amasIon SL, Bremen, Germany) analysis. The amounts of petroleum ether, ethyl acetate and n-butanol fractions of ethanol extract of *Herpesimplex varicella* were 31.5 g, 25.7 g and 15.3 g, respectively. In order to achieve an efficient isolation of target compound, silica gel column chromatography using methanol-chloroform gradient solvent system was used to separate the ethyl acetate fraction into 18 sub-fractions. According to pre-experimental results, the 10th sub-fraction was further isolated to yield 170 mg HPT powder as a white amorphous solid. The structural identification of HPT was carried out by MS, 1H NMR and 13C NMR spectra (Fig. 1) as follows: ESI-MS m/z: 569.2, 584.2, 701.4, 716.4, 983.6 (m/z: 5.0, 6.0, 10.0, 11.0, 18.0) [M+Na]+, [M+K]+. 

**4.4. Preparation and formulation optimization of liposomal HPT**

Liposomal HPT was prepared with phospholipids, cholesterol and HPT by three methods, such as injection method, film dispersion method and anti-solvent evaporation method. With the higher entrainment efficiency (EE) of HPT, film dispersion method was chosen. Briefly, phospholipids, cholesterol and HPT were dissolved into chloroform in a round-bottom flask, in which the lipid film was formed by eliminating the organic solvent under reduced pressure. After the thin film was swollen in F68 solution with a certain concentration for 30 min, the dispersion was sonicated in a water bath for 30 min. After that, the liposomes were filtered through a 0.22 μm filter.

Topographical liposomal HPT with high EE, the influence of main experimental factors on EE was evaluated by a single factor experiment, such as the concentration of chitosan and sonic time. According to the previous method with improvement (Xie et al., 2006), the separation of HPT was established using BEH C18 column (5 μm, 150 × 4.6 mm, 250°C), under the gradient elution of acetonitrile:water (50:50). The injection volume was 20 μl and the flow rate was 1 ml/min. The injection volume was 20 μl and the total run time of each sample was 5 min. The regression equation of peak area versus concentration for HPT standard solutions in methanol was 15.0 ± 0.12 (μg/ml) = 5.5 ± 0.096 (μg/ml) = 0.99999.

Freeze-drying of liposomal HPT.

To ensure long-term stability of liposomes, various lypotectants, such as glucose, mannitol, lactose, and treos, were used in a combination added to the freshly prepared liposomal HPT for optimizing the freeze-drying formulation. The dispersion was freeze-dried in aliquots of 2 ml in 10 ml glass vials. After freezing at −40°C for 3 h, the vials were placed into MODULY-230 freeze dryer (Thermo Scientific, MA, USA) for 24 h under the pressure less than 1 Pa. All vials were sealed with rubber stopper and aluminium seals after lyophilization and stored at 4°C until further treatment. Prior to the measurement of particle size and EE, the freeze-drying powder was reconstituted with distilled water to the original volume.

**4.5. Particle size and morphology of liposomal HPT**

The mean particle size and size distribution of liposomal HPT were measured by a Zetasizer Nano ZS90 (Malvern, Worce, UK). EE was evaluated by the method in section 2.4. Transmission Electron Microscopy (TEM) image of liposomal HPT was obtained using a JEM-100CX TEM microscope (Tokyo, Japan). A staining technique with 0.5% uranyl acetate was adopted to observe the liposomal HPT.

**4.6. Release profile in vitro**

HPT release from liposomes in vitro was also studied. 20 ml of liposome solution was poured on dialysis membrane (MWCO: 3500 Da) and dialyzed against phosphate buffer solution (PBS, pH 7.0, 100 ml) with stirring speed of 100 rpm at 37°C. At specified time intervals, 1 ml of receptor phase was removed and replaced with an equal volume of prewarmed fresh PBS. The released HPT was determined using UPLC and the release data were processed mathematically to deduce the mechanism of HPT release from liposomes.

**4.7. Intravenous injection safety**

To preliminarily clarify the intravenous injection safety, ear vein irritation, hemolysis and coagulant activity were studied. Rabbits weighing 1.8~2.2 kg were involved in the assay. Briefly, 0.5 ml of liposomal HPT was injected into the ear vein with a daily dose of 20 mg/kg. As a control, an equal volume of physiological saline was administered into the left ear marginal vein. After injection for 4 days respectively, the rabbits were sacrificed and the vascular tissues of injection site were cut down and preserved in 10% formalin (Zhao et al., 2010).

Fresh rabbit blood with acid-citrate decahydrate as anticoagulant agent was used to test the hemolysis effect of liposomal HPT. The erythrocytes were collected and washed with physiological saline for three times after centrifugation of whole blood at 2000 rpm for 10 min. Erythrocytes were resuspended by adding saline to obtain 2% hematocrit erythrocyte suspension without fibrinope. Different volumes of liposomal HPT were mixed with 1 ml of erythrocyte suspension to achieve final concentrations of 2%, 4% and 6% (w/v). Physiological saline served as normal control and distilled water was used as 100% hemolysis control, respectively. All the samples were kept at 37°C for 30 min. After that, the samples were centrifuged at 2000 rpm for 10 min to obtain the supernatant, which was determined at 542 nm OD using a Varian 5000 UV spectrophotometer. The percent hemolysis was calculated using the following equation (Zhang et al., 2008).

\[ \text{hemolysis} = \frac{\text{OD}_{542 \text{nm, sample}} - \text{OD}_{542 \text{nm, control}}}{\text{OD}_{542 \text{nm, control}}} \times 100\% \] (1)

The effects of liposomal HPT on whole blood clotting time were determined as follows: freshly collected citrated rabbit blood was mixed 101 (v:v) with liposome solution or saline (control) in the glass vials. Then the 100 mM calcium chloride solution was added into the mixture with the ratio of 1:10 (v:v). At the arranged time point, 3 ml of preheated distilled water at 37°C was infused into the vials (Koziara et al., 2005). Five minutes later, the samples were centrifuged at 2000 rpm for 10 min and the supernatant was obtained and measured at 542 nm in a Cary50 UV spectrophotometer (Varian, CA). The whole blood clotting OD curve was drawn.

**4.8. Animals and experimental design**

Fifty ICR mice were randomly divided into five groups with ten mice in each group. The normal group received a single dose of olive oil (20 mg/kg) intraperitoneally (i.p.) without any treatment or CCI4 injury. Before CCI4...
treatment, mice in the control group received saline by i.v. injection. The HPT control group was treated with free HPT (dissolved in 10% glycerol aqueous solution of 20 mg/kg) as a positive control. The mice in the HPT control group received saline by i.v. injection; the positive group with Qing Kai Ling injection of 20 mg/kg, the positive Chinese medicine with known anti-inflammatory properties; and the liposomal HPT group with liposome dispersion at an equivalent dose of HPT. The administration in these three groups was done for 7 consecutive days. One hour after the last administration, all mice were injected i.p. with 0.1% CCl4 in olive oil (v/v, 20 ml/kg) except the mice in the HPT control group, which were injected i.p. with the equivalent dose of HPT dissolved in 0.1% CCl4 in olive oil. The administration in these three groups was done for 7 consecutive days. One hour after the last administration, all mice were injected i.p. with 0.1% CCl4 in olive oil (v/v, 20 ml/kg). The administration in these three groups was done for 7 consecutive days. One hour after the last administration, all mice were injected i.p. with 0.1% CCl4 in olive oil (v/v, 20 ml/kg) except the mice in the normal group. 16 h later, all mice were sacrificed and the blood was collected for the test of serum ALT, AST and ALP. The liver was removed immediately and a part of the organ was processed for histopathological examination. Another portion of the liver was homogenized with corresponding buffer of kits followed by centrifugation at 2000 rpm for 10 min. The supernatant was then collected for the measurement of MDA and SOD levels.

4.1.1. Data analysis

The data were analyzed with SPSS 15.0 statistical software (SPSS Inc., IL). Multiple comparisons were performed by ANOVA. A value of p < 0.05 was considered statistically significant. The release profile was modeled by Origin 8.0 software (OriginLab Corporation, MA).

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