The aim of the present study was to investigate the effect of various transmembrane ammonium salt gradients and different lipid composition on the loading efficiency of liposomal formulations of irinotecan hydrochloride (CPT-11), their behavior in vivo and cytotoxicity. Among ammonium salts studied, ammonium sulfate was successfully used to load CPT-11 into liposomes with the highest encapsulation efficiency. Subsequently, liposomal CPT-11 with different lipid composition was prepared by ammonium sulfate gradient method. CPT-11 can be loaded to a level over 90% into liposomes composed of soybean phospholipids/cholesterol (SPC-L) or hydrogenated natural soybean phospholipids/cholesterol (HSPC-L). In vitro release profiles were also investigated, indicating that HSPC-L had a lower release than that in SPC-L. In vivo, encapsulation of CPT-11 in both liposomal formulations showed higher area under the curve (AUC), a lower rate of clearance (CL) and smaller volume of distribution for CPT-11 than those of irinotecan hydrochloride solution (CPT-11-S). However, CL and AUC of 7-ethyl-10-hydroxycamptothecin (SN-38) were moderately improved in HSPC-L group. Based on the results of comparative pharmacokinetics of liposomal CPT-11 with different lipid composition, the in vitro cytotoxicity of HSPC-L was evaluated with human tumor cell. The result indicated that liposomal CPT-11 showed a great enhancement in vitro cytotoxicity. The results suggested that entrapment of CPT-11 in liposomes especially in those with high phase-transition temperature lipid by ammonium sulfate gradient would be a promising formulation with a better in vivo behavior.
1. Introduction

Irinotecan (CPT-11), a topoisomerase I inhibitor, has excellent antitumor activity against a variety of human xenografts [1–4]. The clinical introduction of CPT-11 had a significant impact on cancer treatment, particularly colorectal adenocarcinoma and small-cell lung cancers [5–9]. However, CPT-11 is discovered to have serious side effects such as myelosuppression and gastrointestinal disorders (mainly diarrhea), which are recognized as constituting dose-limiting toxicity for this drug [10,11]. CPT-11 has a complicated pharmacologic profile and is extensively metabolized in vivo to yield a number of derivatives including the potent metabolite SN-38 which has 100- to 1000-fold more potent antitumor. Furthermore, it is worth mentioning that CPT-11 requires conversion to SN-38 for optimal activity yet must avoid inactivation via simple hydrolysis of the requisite lactone configuration to an inactive carboxylate. After intravenous administration of free CPT-11, less than 5% of CPT-11 is converted to SN-38, mainly in the liver. Use of drug delivery technologies focused on strategies to stabilize the lactone ring of CPT-11 [12,13]. Therefore, it is desired to find an effective drug delivery system to reduce toxicity and preserve the active form of the drug.

Recent reports have asserted that the liposomal formulations of CPT-11 can reduce the clearance of CPT-11 in vivo and increase antitumor activity [14,15]. The liposomal CPT-11 stabilized the lactone ring of CPT-11 as well. The physicochemical characteristics of CPT-11 make it amenable to efficient encapsulation in pharmaceutically viable liposome systems. CPT-11 can be trapped into liposomes with high encapsulation efficiency by pH-gradient, however, the acidified liposome can introduce instability by hydrolyzing lipid at acidic pH during long-term storage. Recently, a loading method was developed using copper adjusted to neutral pH with triethanolamine (TEA) without generation of a pH-gradient [16,17], whereas there is a possibility that serum copper causes systemic toxicity [18–20].

The transmembrane ammonium salts gradient approach differs from most other chemical approaches used for remote loading of liposomes, since it neither requires preparation of the liposomes in acidic pH, nor to alkalize the extra-liposomal aqueous phase. The stability of the ammonium ion gradient is related to the low permeability of its counterion, the anion which also stabilizes CPT-11 accumulation for prolonged storage periods. It can eliminate systemic toxicity caused by metal ion and load CPT-11 into liposomes with high encapsulation efficiency.

Now we developed a transmembrane ammonium ion gradient extensively in loading the amphipathic weak bases into liposomes to achieve high encapsulation efficiency. As for this method, we investigated the effect of different lipid composition and various transmembrane ammonium salt gradients on the loading efficiency of liposomal formulations. Characterizations of prepared liposomes in vitro were mainly measured. Meanwhile, comparative pharmacokinetics of CPT-11 liposomal formulations and CPT-11 injection (Sol) in rats were carried out. Nevertheless, few published reports have investigated in depth the pharmacokinetics of liposomal CPT-11 encapsulated by ammonium salt gradient method. Also, the study on in vitro cytotoxicity of liposomal CPT-11 was conducted.

2. Materials and methods

2.1. Materials

CPT-11 and camptothecin were obtained from Chengdu Lanbei Plant Chemical Science and Technology Co., Ltd (Chengdu, China). SN-38 was bought from Shanghai Junjie Biotechnology Co., Ltd (Shanghai, China). Hydrogenated natural soybean phospholipids (HSPC) and soybean phospholipids (SPC) were purchased from Lipoid Co. (Germany). All other reagents were of analytical grade and used as received.

2.2. Animals

Wistar male rats weighting between 180 and 220 g were used for pharmacokinetic study. The animals were provided by Experimental Animal Center of Shenyang Pharmaceutical University (Shenyang, China). All experimental procedures were carried out in accordance with the guidelines of the Experimental Animal Care and Use Committee of Shenyang Pharmaceutical University. The rats were fasted overnight before the experiments and water was available ad libitum throughout the experiments.

2.3. Synergistic effect of partition coefficient and different salt gradients on the loading efficiency of CPT-11 into liposomes

1-Octanol was used to represent the biomembrane. The partition coefficients of CPT-11 between 1-octanol and salt solutions at 25 °C were determined by the shake-flask method. Various salt solutions (200 mM), including ammonium salts of sulfate ((NH₄)₂SO₄), phosphate (NH₄-PA), edetate (ETDA-2NH₄), pyrophosphate (NH₄H₂PO₄), polyphosphate (NH₄-APP), chloride (NH₄Cl), citrate (NH₄-CA), and acetate (NH₄-AC), triethanolammonium salts of phytate (TEA-PA) and citrate (TEA-CA), and copper sulfate (CuSO₄), were investigated in this study. 1-Octanol and salt solution were co-saturated with each other for 24 h at 25 °C prior to use. 1-Octanol (4 volumes) and salt solution containing irinotecan (1 mg/ml) (1 volume) were mixed in the screw-capped glass scintillation vials and equilibrated under constant shaking at 25 °C for 24 h. The aqueous phase was then separated by centrifuging at 3000 rpm for 10 min and the concentration of CPT-11 in this phase was analyzed by HPLC. The 1-octanol/salt solution partition coefficients for CPT-11 were calculated.

The solutions of ammonium salts of ETDA-2NH₄, NH₄H₂PO₄, NH₄-APP, NH₄-PA, and NH₄-CA were prepared by titration of edetic acid, pyrophosphoric acid, polyphosphoric acid, phytic acid, and citric acid solutions with concentrated ammonia and diluted to a final concentration of 200 mM, respectively. Also, the solutions of triethanolammonium salts of TEA-PA and TEA-CA were prepared by titration of phytic acid and citric acid solutions with TEA and diluted to a final concentration of...
200 mM, separately. The rest salt solutions were prepared by dissolving corresponding commercial salts in water.

Meanwhile, various salts mentioned above were used to prepare CPT-11 liposomes by transmembrane salt gradient (shown in section 2.4) and encapsulation efficiency measurements were performed as described in section 2.5.2.

2.4. Preparation of CPT-11 containing liposomes

Lipids (SPC/Chol or HSPC/Chol) were dissolved in chloroform in a round bottom flask at the required ratio and dried to form a thin film under reduced pressure on a rotary evaporator, which was placed in a high vacuum to remove any residual solvent. Subsequently, the lipid films were hydrated with salt solution studied above to achieve multilamellar vesicles (MLVs) with a final lipid concentration of 100 mg/ml. The hydrated temperature of SPC/Chol or HSPC/Chol is 40 °C and 55 °C respectively. Following MLVs were sonicated by a probe tip sonicator and then passed through the filter of 0.22 μm pore size, to thereby prepare large unilamellar vesicles (LUVs). CPT-11 encapsulation into LUVs was achieved by remote loading method as followed: the external aqueous phase of LUVs was exchanged, using Sephadex G-50 size exclusion chromatography, with a 10 mM histidine/10% sucrose solution. The preformed liposomes were prepared. The CPT-11 solution was added to the preformed liposomes and incubated at 60 °C for 10 min. After introduction, the sample was cooled in ice for 15 min. The liposome dispersions were passed through a 0.22 μm membrane to eliminate any microbes and then stored at 4–8 °C.

2.5. Characteristics of liposomal CPT-11

2.5.1. Particle size measurement

The particle size of prepared liposomes was measured by dynamic light scattering using NICOMP 380 submicron particle size analyzer (PSS, Santa Barbara, CA, USA) with a scattering angle of 90°. The samples were diluted with physiological saline to suitable optical density value prior to measurement.

2.5.2. Encapsulation efficiency measurement

The separation of liposomes from free CPT-11 was performed by size exclusion chromatography using a Sephadex G-50 column. After separation, the amount of CPT-11 in liposomes was determined at 370 nm by ultraviolet spectrophotometer (Unicomp Instrument Co., Ltd, China) after lysis of the liposomes with Triton X-100. Then the total amount of CPT-11 in liposomal suspension was assayed by the same method mentioned above. The encapsulation efficiency was calculated.

2.5.3. In vitro release experiments

Because of the easy conversion of CPT-11 into carboxylate form in neutral medium, PBS with pH 5.0 was chosen as the release medium in release experiments in vitro. The release experiments were carried out in an isotonic PBS buffer with pH 5.0 at 37 °C. Briefly, 2.5 ml HSPC-L (equivalent to 10 mg CPT-11) was placed in a dialysis membrane bag with a molecular weight cut-off of 10 kDa, and immersed in 500 ml of PBS medium. The entire system was kept at 37 °C and stirred at a rate of 100 rpm. At predetermined time intervals, 3 ml of samples were collected, and the amounts of released CPT-11 from liposomes were determined at 370 nm by ultraviolet spectrophotometer, and the accumulated release rate was calculated. Each release experiment was run three times. The release experiment of SPC-L was conducted by the same method. Bring into comparison, the release of CPT-11 solution from the dialysis bag under the same conditions was also evaluated. The zero order kinetic equation, first-order kinetic equation, Higuchi’s equation, and Peppas’s equation were applied to fit release data.

Simultaneously, the release experiment of HSPC-L with rat plasma was investigated. The release experiment of CPT-11 solution with rat plasma was also conducted as control group. Briefly, equal volumes of the sample and rat plasma were mixed prior to the experiments. Subsequently, experiments were carried out according to the method mentioned above, except that the amounts of released CPT-11 from liposomes were determined by HPLC.

2.5.4. Conversion of liposomal CPT-11 to SN-38 in vitro

CPT-11 liposomes and rat plasma (1:8, v/v) were mixed together and incubated at 37 °C. At indicated time points, the concentration of SN-38 was evaluated by HPLC. The conversion rate was calculated by the molar ratio of SN-38 to CPT-11 (initial molar). The conversion of CPT-11 solution to SN-38 was also investigated. The experiments were repeated three times.

2.6. Pharmacokinetic study of CPT-11 liposomes

2.6.1. Analysis of CPT-11 and SN-38 in plasma

A simple HPLC method with fluorescence detection was developed for the determination of CPT-11 and SN-38 in rat plasma. The HPLC system (Jasco Corporation, Japan) consisted of PU-1580 pump with L-7485 fluorescence detector (Hitachi Co., Japan). HPLC separation was performed in a stainless-steel Diamonsil C18 column (150 × 4.6 mm I.D.; 5 μm particle size; Dikma) and the column temperature was maintained at 30 °C. The mobile phase consisted of acetonitrile-50 mM disodium hydrogen phosphate (30:70, v/v) containing 5 mM heptanesulphonate, adjusted to pH 3.0 with orthophosphoric acid. The flow-rate was set at 1.0 ml/min and the injection volume was 10 μl. The excitation wavelength was set at 556 nm, and the emission wavelength was 380 nm. Both concentrations of CPT-11 and SN-38 were calculated from the ratio of their peak areas to that of the I.S. Additionally, linearity, extraction recovery, precision and accuracy were carried out in method validation. The working solutions of CPT-11, SN-38 and CPT-I.S. were prepared according to Hidetaka Sumiyoshi et al. [21].

2.6.2. Drug administration and sample preparation

Wistar rats were randomly divided into three groups, the HSPC-L, SPC-L, and Sol group and each group containing five rats. Each group was administrated at a dose of 20 mg/kg via the tail vein. After dosing, blood samples were collected at predetermined time intervals. Plasma was separated immediately by centrifugation with low temperature and 100 μl was collected for analysis by HPLC. Briefly, 100 μl I.S. solution (CPT, 1 μg/ml) and 300 μl acidified methanol (methanol-hydrochloride (9:1), v/v) were added to 100 μl plasma sequentially. All solutions were cooled in
ice before addition. Samples were then vortex-mixed for 1 min and separated by centrifugation at 14,000 rpm for 10 min. Finally, the 10 µl supernatant was injected into the HPLC system.

2.6.3. Data analysis
The data were analyzed by DAS version 2.0 software (Mathematical Pharmacology Professional Committee of China, Shanghai, China). The main pharmacokinetic parameters were calculated. A statistical comparison of the pharmacokinetic parameters was conducted using Student’s t-test and one-way analysis of variance (ANOVA). The significance level was set to be 0.05.

2.7. In vitro cytotoxicity
Hela (cervical), Hos (osteosarcoma), BGC-823 (stomach), MCF-7 (breast) were used to investigated the in vitro cytotoxicity of liposomal CPT-11. The cells were maintained in RPMI1640 culture medium (Hos in MEM) in tissue culture flasks in a humidified incubator at 37 °C in an atmosphere of 95% air and 5% CO2. Medium was changed 3 times a week and cells were passaged using trypsin/EDTA. For dose-dependent cytotoxicity assays, Cells were seeded in 96-well plates at 3000 cells/well with 3 replicates and pre-incubated for 24 h. Media were replaced with fresh medium. And then predetermined amounts of CPT-11 and liposomal CPT-11. The in vitro cytotoxicities of CPT-11 and liposomal CPT-11 were determined using MTT assay.

3. Results and discussion

3.1. Synergistic effect of partition coefficient and different salt gradients on the loading efficiency of CPT-11 into liposomes

Generally, it was considered that the partition coefficient of drug in 1-octanol and water (P-value) is an index to estimate whether the drugs can be encapsulated in liposomes easily or not. The hydrophilic (Log \( P < -0.3 \)) or lipophilic (Log \( P > 4.5 \)) drugs can be encapsulated in liposomes successfully and show the stable physicochemical characteristics [22]. Though the Log \( P \) octanol/water for CPT-11 was lower than −0.3 (shown in Table 1), CPT-11, a drug of amphipathic weak base, can not be trapped into liposomes with high encapsulation efficiency by passive loading method. As the partition coefficient of amphipathic weak base between 1-octanol and water was prone to be influenced by the pH and ionic strength of medium. Thus, CPT-11 was a good candidate to be trapped into liposomes by remote loading methods. The octanol/medium partition coefficient and state of the drug in intraliposomes may serve as crucial factors affecting the loading efficiency of drug into liposomes [23,24]. Thus, the effect of octanol/salt solution partition coefficient for CPT-11 and type of anion of salt on the encapsulation efficiency of CPT-11 liposomes were both investigated in this study.

As shown in Table 1, the Log partition coefficient (Log \( P \)) values for CPT-11 ranging from 0.21 to 1.50, indicating that the sort of salt solution could affect on the octanol/salt solution partition coefficient. Additionally, the Log \( P \) for CPT-11 in octanol/ammonium sulfate solution was the smallest, and the encapsulation efficiency was the highest. Whereas, no regular relationship was observed between the Log \( P \) values and the encapsulation efficiency. It was suggested that partition coefficient for CPT-11 was not the only factor affecting the encapsulation efficiency of liposomes in remote loading method. Ammonium salt anion, another factor, was found to influence on the encapsulation efficiency and the leakage of CPT-11 liposomes.

The role of ammonium salt anion is not only loading the amphipathic weak base but rather to control the stability of loading and the release rate of the amphipathic weak base from the liposomes to the external aqueous phase. Two major factors may influence the role of the ammonium salt anion. Firstly, their ability to induce precipitation/crystallization/gelation in the intraliposome aqueous phase, and secondly, their effect on the membrane/buffer or 1-octanol/buffer partition coefficient of the amphipathic weak base [24]. Among low molecular weight anions (inorganic and organic) used to achieve ammonium ion-drive loading, the order of loading stability for most amphipathic weak bases studied is sulfate > citrate > phosphate > chloride > glucuronate [25,26]. However, the results (shown in Table 1) in this study demonstrated that the order of loading stability for CPT-11 was sulfate > EDTA > phosphate > chloride > citrate > acetate, which might be concerned with the property of the drug.

On the other hand, the encapsulation efficiency of liposomes prepared by triethanolammonium salts gradient was

<table>
<thead>
<tr>
<th>Octanol/aqueous solutions</th>
<th>Partition coefficients (P)</th>
<th>Log ( P )</th>
<th>Encapsulation efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Octanol/ammonium polyphosphate</td>
<td>31.27</td>
<td>1.50</td>
<td>81.67</td>
</tr>
<tr>
<td>Octanol/ammonium pyrophosphate</td>
<td>21.97</td>
<td>1.34</td>
<td>64.39</td>
</tr>
<tr>
<td>Octanol/ammonium phytate</td>
<td>15.84</td>
<td>1.20</td>
<td>84.50</td>
</tr>
<tr>
<td>Octanol/ammonium chloride</td>
<td>10.16</td>
<td>1.01</td>
<td>79.18</td>
</tr>
<tr>
<td>Octanol/ammonium EDTA</td>
<td>9.91</td>
<td>0.996</td>
<td>87.50</td>
</tr>
<tr>
<td>Octanol/ammonium citrate</td>
<td>8.57</td>
<td>0.93</td>
<td>16.20</td>
</tr>
<tr>
<td>Octanol/phytic acid (adjust pH to 6.5 with TEA)</td>
<td>7.26</td>
<td>0.86</td>
<td>4.16</td>
</tr>
<tr>
<td>Octanol/ammonium acetate</td>
<td>5.76</td>
<td>0.76</td>
<td>4.38</td>
</tr>
<tr>
<td>Octanol/citric acid (adjust pH to 6.5 with TEA)</td>
<td>4.64</td>
<td>0.67</td>
<td>2.42</td>
</tr>
<tr>
<td>Octanol/citric acid (adjust pH to 6.5 with TEA)</td>
<td>4.64</td>
<td>0.67</td>
<td>2.42</td>
</tr>
<tr>
<td>Octanol/CuSO4</td>
<td>1.72</td>
<td>0.24</td>
<td>80.53</td>
</tr>
<tr>
<td>Octanol/ammonium sulfate</td>
<td>1.63</td>
<td>0.21</td>
<td>90.42</td>
</tr>
<tr>
<td>Octanol/water</td>
<td>0.355</td>
<td>−0.45</td>
<td>−</td>
</tr>
</tbody>
</table>
promote drug loading into liposomes [28]. However, the transition metal copper and then such complexation can intraliposomal aqueous phase; the CPT-11 can complex the lower solubility of the CPT-11 sulfate salt formed in the copper sulfate gradient. The reasons may be as follows: the CPT-11 liposomes can be also obtained by transmembrane formed [27]. Therefore, transmembrane ammonium ion gradient was used to entrap the CPT-11 into liposomes.

Furthermore, high encapsulation efficiency (80.53%) of CPT-11 liposomes can be also obtained by transmembrane copper sulfate gradient. The reasons may be as follows: the lower solubility of the CPT-11 sulfate salt formed in the intraliposomal aqueous phase; the CPT-11 can complex the transition metal copper and then such complexation can promote drug loading into liposomes [28]. However, the toxicity of transition metal was inscrutable in vivo.

Base on these observations, lower octanol/salt solution partition coefficient and lower permeability coefficient of the anion through lipid bilayers were crucial factors in obtaining intraliposomal accumulation of CPT-11. An additional reason for having stable CPT-11 accumulation is the lower solubility of the CPT-11 salt formed in the intraliposomal aqueous phase. The \( \log k \) for CPT-11 in octanol/ammonium sulfate solution was the smallest, and the encapsulation efficiency was the highest. Therefore, transmembrane ammonium sulfate gradient method was chosen to prepare the CPT-11 liposomes. The encapsulation efficiency of CPT-11 liposomes in this study could reach over 90%.

3.2. Encapsulation efficiency and particle size distribution of liposomes

CPT-11, a drug of amphipathic weak base (\( pK_a = 8.1 \)), can be trapped into liposomes with high encapsulation efficiency by remote loading methods including pH-gradient, transmembrane ammonium ion gradient, and transition metal ion gradient [29–32]. Because of some disadvantages in pH-gradient and metal ion gradient [28,33], transmembrane ammonium ion gradient has been developed extensively in loading the amphipathic weak bases into liposomes to achieve high encapsulation efficiency. In this paper, transmembrane ammonium ion gradient was used to entrap the drug into liposomes.

The encapsulation efficiency of SPC-L and HSPC-L was 91.68% and 95.23%, respectively. It is thus clear that CPT-11 can be trapped into liposomes with high encapsulation efficiency by transmembrane ammonium ion gradient method. The mean diameter of SPC-L and HSPC-L possessed 92.2 ± 45.9 nm, 98.3 ± 52.2 nm, individually, measured by dynamic light scattering.

3.3. In vitro release experiments

Accumulated release profiles of CPT-11-S, SPC-L, and HSPC-L were shown in Fig. 1. It could be achieved from the profiles that the accumulated release percentage of CPT-11-S reached 83.13 ± 2.77% at 4 h, while it demonstrated merely 43.57 ± 3.87% and 2.68 ± 0.55%, respectively, for SPC-L and HSPC-L. The presence of the dialysis bag did not significantly affect the release rate of CPT-11. It was obvious that the release of CPT-11 from SPC-L was faster than that from HSPC-L, which could be due to the lower phase-transition temperature of SPC than that of HSPC. It was evident that HSPC-L has a sustained release in vitro.

The accumulated release profiles of CPT-11-S and HSPC-L, both with rat plasma, were shown in Fig. 2. A quicker release behavior of CPT-11 was observed in HSPC-L with rat plasma when compared with that in HSPC-L with PBS. It was thus evident that plasma could accelerate the release of CPT-11 from liposomes. The biological fate of liposomes might be affected by plasma factors. As various plasma components could influence the stability of liposomes and high density lipoprotein (HDL) was the essential one. Apolipoprotein A-1 (apoA-1) was liable to fall off from HDL, and interacted with phospholipids of liposomes. That was to say, exchange of apoA-1 and phospholipids occurred between HDL and liposomes, resulting the formation of hole on the liposomal membranes. Additionally, hydrolysis of phospholipids was caused by phospholipase existing in plasma, and a serum albumin-liposome complex was formed by the interaction of serum albumin with phospholipids of liposomes, which would decrease the stability of liposomes [34]. In addition, the different release patterns were also observed, the best fit with higher correlation coefficient was found in release data of HSPC-L with Peppas’s equation, while the release behavior of HSPC-L at the presence of plasma was fit to the Higuchi’s equation. The release data of SPC-L were best fit to first-order kinetic equation. The equations were shown as follows:

\[
\ln Q = 1.06 \ln t - 0.37, \quad r = 0.9952 \quad \text{(Peppas’s equation)}
\]

\[
Q = 15.67 t^{1/2} - 3.64, \quad r = 0.9969 \quad \text{(Higuchi’s equation)}
\]
Therefore, immediate pretreatments were required for blood of carboxylesterases existing in plasma at high level \[36\].

In the assay, the lower limits of quantitation (LLQ) for CPT-11 and SN-38 were 20 and 2 ng/ml, respectively. The within-day and between-day reproducibility of the assay for CPT-11 and SN-38 were acceptable.

3.4. Conversion of liposomal CPT-11 to SN-38 in vitro

The profiles of the conversion percentage of SN-38 from CPT-11 in rat plasma in vitro for HSPC-L and CPT-11-S were shown in Fig. 3. It was indicated that the conversion of SN-38 from CPT-11 in HSPC-L was slightly less than that in CPT-11-S group, but there were no significant statistical differences \((P > 0.05)\) between the two groups. It was suspected that the conversion of SN-38 from CPT-11 also existed in the membranes of liposomes, which have been reported by Y. Sadzuka \[35\]. Though the conversion of SN-38 occurred, SN-38 was still entrapped in the intraliposomes. In this way, SN-38 could be refrained from metabolization by enzyme systems in plasma and high concentration of SN-38 was maintained simultaneously.

3.5. Pharmacokinetic study of CPT-11 liposomes

3.5.1. Assay validation

HPLC-Fluorescence method was used for the determination of CPT-11 and its metabolite SN-38 in plasma, and the results of validation were as follows:

The CPT-11, SN-38 and CPT were extracted from plasma by a methanol extraction, and their recoveries were over 90%. The mean ± SD absolute recovery of the I.S. (CPT 1 μg/ml plasma) was 99.33 ± 1.74%. The range of selected CPT-11 concentration was so wide that two standard curves of CPT-11 were established. The standard curve was established by plotting the ratio of the peak area to that of CPT against the concentration. Good standard curves were obtained for CPT-11 and SN-38, ranging from 20 to 1000 ng/ml, 600–24,000 ng/ml, and 2–200 ng/ml, respectively, which were sufficient for the pharmacokinetic studies of CPT-11. The lower limits of quantitation (LLOQ) for CPT-11 and SN-38 were 20 and 2 ng/ml, respectively. The within-day and between-day reproducibility of the assay for CPT-11 and SN-38 were investigated. Both the precision and accuracy of this method were acceptable.

CPT-11 was easy to be converted into SN-38 in the presence of carboxylesterases existing in plasma at high level \[36\]. Therefore, immediate pretreatments were required for blood samples, which can inhibit the conversion of CPT-11 into SN-38 in plasma, so that the concentrations of CPT-11 and SN-38 in plasma can be determined with high accuracy. In addition, the aim of acidifying samples was to transform the carboxylate forms of CPT-11 and SN-38 to the closed lactone forms, and determine the total concentrations of CPT-11 and SN-38 in plasma.

3.5.2. Pharmacokinetics

The pharmacokinetic behavior of CPT-11 liposomes was investigated and compared with that of CPT-11 solution. Also, comparative pharmacokinetics of SPC-L and HSPC-L were conducted.

The plasma concentration versus time profiles of CPT-11 and SN-38 obtained after i.v. injection of SPC-L, HSPC-L, and Sol in rats were showed in Figs. 4 and 5, respectively. As shown in Figs. 4 and 5, there were several striking differences in pharmacokinetic properties between the liposomal CPT-11 formulations and CPT-11 solution. In the plasma, the CPT-11 concentrations at 5 min, 15 min, 0.5 h, and 1 h after injection of the SPC-L group increased 12.5-fold \((P < 0.01)\), 6.3-fold \((P < 0.01)\), 3.2-fold \((P < 0.01)\), and 2.1-fold \((P < 0.01)\), respectively, compared with that in the Sol group, but there were no significant differences ranging from 1.5 h to 12 h \((P > 0.05)\).

CPT-11 concentrations in plasma were below the detection limit at 24 h after dosing in both groups of SPC-L and Sol. In addition, the plasma concentrations of SN-38 were nearly the same \((P > 0.05)\) at all indicated time between the SPC-L and Sol. Nevertheless, the plasma concentrations of CPT-11 were higher in HSPC-L than that in Sol or SPC-L at all time points. The CPT-11 concentration in plasma at 5 min, 15 min, 0.5 h, and 1 h after injection of the HSPC-L group increased 70-fold \((P < 0.01)\), 57-fold \((P < 0.01)\), 43-fold \((P < 0.01)\), and 38-fold \((P < 0.01)\), respectively, higher than that in the Sol group. Moreover, the plasma concentrations of SN-38 were higher in HSPC-L than that in Sol or SPC-L at all time points especially ranging from 2 to 24 h.

The main pharmacokinetic parameters were shown in Table 2. It was exhibited that HSPC-L had a 32.6-fold \((P < 0.01)\) increase in the AUC of CPT-11 when compared to Sol, while AUC for SPC-L was 3.3-fold \((P < 0.01)\) greater than that of Sol.

![Fig. 3](image1)  The profiles of conversion percentage of SN-38 from CPT-11 in vitro for HSPC-L and CPT-11-S in rat plasma.

![Fig. 4](image2)  Plasma concentration of CPT-11 versus time curves after intravenous administration of HSPC-L, SPC-L and Sol, respectively \((n = 5, \text{ mean } \pm \text{ SD})\).
The main pharmacokinetic parameters of CPT-11 and SN-38 after i.v. administration of HSPC-L, SPC-L and Sol to rats, respectively (n = 5).

![Fig. 5 — Plasma concentration of SN-38 versus time curves after intravenous administration of HSPC-L, SPC-L and Sol, respectively (n = 5, mean ± SD).](image)

The CL and distribution volume (V) of CPT-11 in SPC-L group was 3.2 (P < 0.01) and 3.6 times (P < 0.01), respectively, lower than that in the Sol group. Furthermore, it was indicated that HSPC-L had a 29.5-fold and 29.4-fold decrease in the CL and V of CPT-11, respectively, compared with the Sol group. On the other hand, the t_{1/2} of CPT-11 did not differ significantly between the liposomal formulations (HSPC-L, SPC-L) and solution (P > 0.05). Compared with SPC-L, HSPC-L exhibited 10.5-fold higher AUC of CPT-11, 8.8-fold lower of CL (P < 0.01), and 8.2-fold lower of V (P < 0.01). The pharmacokinetic parameters of SN-38 were comparable in both groups of SPC-L and Sol (P < 0.05). However, HSPC-L demonstrated 3.03- and 3.8-fold higher AUC of SN-38 (P < 0.05), respectively, than those in Sol and SPC-L group. Meanwhile, HSPC-L had a 2.57- and 3.12-fold decrease in clearance of SN-38 (P < 0.05), when compared with Sol and SPC-L group, respectively.

It was concluded that liposomes, as carriers of CPT-11, can significantly improve AUC, decrease CL and V of CPT-11. HSPC-L demonstrated superior pharmacokinetic behavior of CPT-11 or SN-38, when compared with SPC-L or Sol.

In vivo pharmacokinetics behavior differences between free and liposomal CPT-11 might be due to the differences of their stability in vivo. Compared with liposomal CPT-11, the free one, after i.v. administration, eliminates fast by hydrolysis in plasma, transport to surrounding tissues and its elimination via the kidney. Simultaneously, owing to its encapsulation in the inner phase of liposomes, liposomal CPT-11 could escape hydrolysis in vivo after administration, which would result in higher plasma concentration of CPT-11 in active form. The following possible reasons can be explained for the different in vivo behaviors between HSPC-L and SPC-L. Liposomes with different lipid composition should have different in vivo behavior. The phase-transition temperature (about –20 °C) of SPC was lower than body temperature (37 °C), which would decrease the stability of SPC-L after administration, resulting in quick release of CPT-11. On the contrary, HSPC with high phase-transition temperatures (about 50 °C) should slow the leakage of CPT-11 from intraliposomes and warrant high drug level in blood circulation system.

On the other hand, the conversion of SN-38 occurring in CPT-11 liposomes (as shown in Fig. 3) and the pharmacokinetic behavior of CPT-11 liposomes, suggested that CPT-11, a pro-drug, can also be trapped effectively in liposomes. However, the blood circulation of CPT-11 was not improved greatly by liposomal formulation. The order of elimination t_{1/2} value for CPT-11 and SN-38 was HSPC-L > Sol > SPC-L, but there is no significant difference between those values, suggested that the elimination of CPT-11 liposomes from blood circulation is still rapid. It might be due to that unmodified CPT-11 liposomes were unavoidable to be recognized by alternative pathways of complement activation and captured by macrophages in RES organs. Therefore, the next study point in this investigation may be the modification on the surface of the liposomes to achieve an improvement in both circulation time and plasma levels of CPT-11 and SN-38.

### 3.6. In vitro cytotoxicity of liposomal CPT-11

The biological activity of liposomal CPT-11 was investigated and compared with that of CPT-11 using Hela, Hos, BGC-823 and MCF-7. Liposome-entrapped CPT-11 showed a great

<table>
<thead>
<tr>
<th>Parameters</th>
<th>AUC_{0→∞} (μg/ml h)</th>
<th>t_{1/2} (h)</th>
<th>V (l/kg)</th>
<th>CL (l/h/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPT-11 (mean ± SD)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSPC-L</td>
<td>306.24 ± 133.17&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.81 ± 0.91</td>
<td>1.7 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.08 ± 0.03</td>
</tr>
<tr>
<td>SPC-L</td>
<td>29.22 ± 4.99&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.36 ± 0.40</td>
<td>1.40 ± 0.60&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.70 ± 0.12</td>
</tr>
<tr>
<td>Sol</td>
<td>2.17 ± 0.59</td>
<td>1.59 ± 0.57</td>
<td>5.04 ± 1.69</td>
<td>2.27 ± 0.66</td>
</tr>
<tr>
<td>SN-38 (mean ± SD)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSPC-L</td>
<td>0.88 ± 0.37&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.52 ± 4.24</td>
<td>243.87 ± 7.13</td>
<td>28.35 ± 17.72&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>SPC-L</td>
<td>0.23 ± 0.05</td>
<td>3.38 ± 0.60</td>
<td>426.96 ± 89.07</td>
<td>88.48 ± 15.40</td>
</tr>
<tr>
<td>Sol</td>
<td>0.29 ± 0.07</td>
<td>4.04 ± 1.43</td>
<td>421.43 ± 175.92</td>
<td>72.73 ± 15.49</td>
</tr>
</tbody>
</table>

<sup>a</sup> P < 0.01 compared to the Sol.
<sup>b</sup> P < 0.05 compared to the Sol.

### Table 2 — The main pharmacokinetic parameters of CPT-11 and SN-38 after i.v. administration of HSPC-L, SPC-L and Sol to rats, respectively (n = 5).

### Table 3 — In vitro cytotoxic activity of liposomal CPT-11 and CPT-11 on different human tumor cells (IC_{50}, n ≥ 3).

<table>
<thead>
<tr>
<th>Cancer cells</th>
<th>Means ± SD</th>
<th>Liposomal CPT-11 (μg/ml)</th>
<th>CPT-11 (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hela</td>
<td>3.118 ± 2.028&lt;sup&gt;a&lt;/sup&gt;</td>
<td>16.08 ± 5.446</td>
<td></td>
</tr>
<tr>
<td>Hos</td>
<td>5.806 ± 3.001&lt;sup&gt;a&lt;/sup&gt;</td>
<td>19.51 ± 6.083</td>
<td></td>
</tr>
<tr>
<td>BGC-823</td>
<td>2.115 ± 0.366&lt;sup&gt;a&lt;/sup&gt;</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>MCF-7</td>
<td>2.807 ± 0.346&lt;sup&gt;a&lt;/sup&gt;</td>
<td>16.265 ± 6.170</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> P < 0.05 compared to the CPT-11.
increased cytotoxicity versus CPT-11. The IC\textsubscript{50} value against Hela, Hos, BGC-823 and MCF-7 were 3.12, 5.81, 2.12 and 2.81 \(\mu\)g/ml for liposomal CPT-11 and 16.08, 19.51, >60, and 16.27 for CPT-11 (show in Table 3). The IC\textsubscript{50} value of 48 exposure to liposome containing CPT-11 is 5.15-fold, 3.36-fold, more than 28.37-fold and 5.79-fold greater than free drug. The increased cytotoxicity derived from encapsulation may arise from preserving the active form of the CPT-11. Because the pH of medium was near physiological pH value and CPT-11 hydrolyzed to yield the inactive carboxyl species. But on the contrary, the internal acidic environment of the liposome limits hydrolysis of the lactone ring of camptothecins to the inactive carboxyl form. The CPT released from the liposomes into the culture medium slowly and maintained the effective concentration of the active form of the CPT-11 for a long time. But hydrolysis of free CPT-11 yielded a high percentage of inactive carboxyl form drug. So greatly enhanced cytotoxicity was obtained by encapsulating CPT-11 in liposome.

### 4. Conclusion

In this study, CPT-11 was entrapped into liposomes successfully by transmembrane ammonium sulfate gradient, possessing high encapsulation efficiency and sustained release in vitro. In vivo, encapsulation of CPT-11 in liposomes showed higher AUC, a lower rate of clearance and smaller volume of distribution for CPT-11 than those in Sol. The rate of clearance and AUC of SN-38 were improved in HSPC-L, but no obvious difference was observed in the pharmacokinetic parameters of SN-38 between SPC-L and Sol. HSPC-L demonstrated superior pharmacokinetic behavior of CPT-11 and SN-38, compared with SPC-L and Sol. The increased cytotoxicity of liposomes may be due to it can preserving more active form of the CPT-11. It appears that entrapment CPT-11 in liposomes especially in those with high phase-transition temperature by transmembrane ammonium salt gradient would be a promising formulation with a better in vivo behavior.

### References


