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ORIGINAL ARTICLE

Sustained release of hydroxycamptothecin after subcutaneous administration using a novel phospholipid complex—DepoFoam™ technology

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Abstract

Objective: In order to prolong the duration of drug in the circulation, multivesicular liposome (MVL, namely DepoFoam™) was utilized as a sustained-release delivery system for hydroxycamptothecin (HCPT). *Methods*: HCPT is insoluble in both water and physiological acceptable organic solvents; therefore, HCPT–phospholipid complex (HCPT–PCC) was prepared by solvent evaporation method to improve its liposolubility. In this study, preparation, characterization, in vitro release, and in vivo pharmacokinetics of HCPT–phospholipid complex-loaded MVLs (HCPT-MVLs) were investigated. *Results*: The results showed that the average particle size of HCPT-MVL was 9 μm and the encapsulation efficiency was 90%. In addition, HCPT-MVLs could improve both in vitro release and in vivo pharmacokinetic behaviors of the original drug, with a sustained release of drugs over 5–6 days. *Conclusion*: These data suggested that by combined use of DepoFoam™ and phospholipid complex formation technique HCPT could be successfully entrapped into the MVLs, which might provide a paradigm for sustained release of insoluble drugs.

Key words: *Hydroxycamptothecin; multivesicular liposomes; phospholipid complex; sustained release*

Introduction

Hydroxycamptothecin (HCPT), as an effective antitumor agent targeting nuclear enzyme topoisomerase I (Top I), has been extensively used in clinic for the treatment of a wide spectrum of cancers such as gastric carcinoma, hepatoma, leukemia, and tumor of head and neck[1]. However, the major barrier for clinical application is its poor water solubility, and the conventional strategy to solve this problem is to administrate HCPT in its carboxylate form. Although the solubility of HCPT is enhanced by this approach, other more severe problems are brought out by the transformation from lactone to carboxylate forms. Generally, the opening of labile E-ring results in immediate metabolism of HCPT, with an extremely short in vivo half-life of 5 minutes[2]. Moreover, because of the destruction of active group of HCPT in carboxylate form, activity of HCPT is significantly reduced with some unpredictable side effects[3].

Previous study demonstrated that HCPT belongs to the class of time-dependent and cell cycle-specific drugs[4]; therefore, multivesicular liposomes (MVLs) or DepoFoam™ particles were assumed to be an ideal dosage form for HCPT with various attracting properties such as prolonged duration of drug in circulation, effective maintenance of the therapeutic drug levels in the blood for a long time, reduced administration frequencies, and therefore increased patient compliance[5,6,7].

MVLs are distinguished structurally from traditional unilamellar vesicles, multilamellar vesicles, and niosomes in that each particle comprises a set of closely packed nonconcentric vesicles. As a result, when a single breach in the outermost membranes of an MVL leads to the release of encapsulated drug to the external medium, the other part remains integrated without the drug getting released from the particle[8]. MVLs show excellent sustained-release features; a number of DepoFoam™-based formulations have presented

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sustained-release patterns from a few days to several weeks[9–22].

Among present studies, most model drugs used with DepoFoam™ technology are water soluble, and there are very few studies concerning poor water-soluble drugs. In addition, most of the phospholipids utilized in DepoFoam™ particles were pure and expensive. Therefore, the main objective of this study was to formulate poor aqueous solubility drug into MVLs with conventional phospholipid such as lipoid E-80 (Lipoid GmbH, Ludwigshafen, Germany). In our study, HCPT remained in a favorable lactone form by the preparation of HCPT–phospholipid complex (HCPT–PCC), by which the lipid solubility of HCPT could be remarkably enhanced[23–25]. Then, HCPT–PCC was incorporated into MVLs with high encapsulation efficiency. Alternatively, emulsifiers were added to improve the appearance and stability of MVLs derived from phospholipids. Consequently, MVLs with a rather high encapsulation efficiency and excellent sustained-release characteristics were obtained, which highlighted the potential use of coupling DepoFoam™ technique with phospholipid complex in the administration of antitumor drugs.

Materials and methods

Materials

A reference standard of HCPT was purchased from Chinese National Institute for the control of pharmaceutical and biological products. HCPT was purchased from Sichuan Guanghan Bio-Technology Co. Ltd. (no. 060208, with a content of 98.29%) (Guanghan, China). Vitamin E (V_E) was purchased from Southwest Synthetic Pharmaceutical Co. Ltd.; phosphatidyl choline (PC, E-80, Lipoid GmbH), Chongqing, China, cholesterol (Chol, purchased from Chengdu Kelong Chemical Plant, Chengdu, China), and soybean oil (SO, from Tieling Beiya Medical Oil Co. Ltd., Tieling, China) were used as lipid phase; anhydrous dextrose (supplied by Tianjin Kemiou Chemical Company, Tianjin, China), glycine (purchased from Tianjin Bodi Chemical Limited Company, Tianjin), and Myrj-59 (polyoxyethylene 100 stearate, obtained from Sigma, St. Louis, MO, USA) were used as emulsifier or addictives in aqueous phase; dialysis bag with a molecular weight cutoff of 8000–12,000 was supplied by Sigma; ultrapure water was used for all solutions and dilution. All the other reagents were of analytical grade and used without further purification.

Preparation of HCPT–PCC

HCPT–PCC was prepared with HCPT, PC, and V_E by solvent evaporation method according to the method developed in our group. Briefly, HCPT, phospholipid at a ratio of 1:2 (mol:mol), and V_E were co-dissolved in acetone, followed by gentle stirring at 50°C for 1 hour until the formation of a clear mixture. Then, acetone was removed on a rotary evaporator (Shanghai Shensheng Technology Co., Ltd., Shanghai, China). Ultimately, the resultant complex was vacuum-dried for 12 hours and kept in a desiccator for subsequent experiments.

Preparation and characterization of HCPT-MVLs

Preparation and optimization of HCPT-MVLs formulations

The HCPT-MVLs were prepared using two-step W/O/W double emulsification process. In brief, a series of formulations with different weight ratios of phospholipids, Chol, SO, and HCPT–PCC were dissolved in 0.5 mL of mixed organic solvent of chloroform and ether (1:1, v/v) and mixed with 4% of anhydrous dextrose solution. Then, the mixture was emulsified on a high shear-force mixer (Shanghai Fluka Electromechanical Equipment Co. Ltd., Shanghai, China) for different time to form a primary emulsion. Subsequentially, the primary emulsion was injected into aqueous solution containing 0.3% of Myrj 59, 1.5% of anhydrous dextrose, and 1.5% of glycine quickly. The mixture was vortexed for 8 seconds by fluid mixer (Jiangxi Medical Apparatus and Instrument Factory, Nanchang, China) to form a W/O/W emulsion. The multiple emulsion was transferred to a 10-mL round bottom flask and the organic solvents were removed on a rotary evaporator (Shanghai Shensheng Technology Co. Ltd.). Different formulation or processing parameters screened for optimal conditions were listed in Table 1.

Particle characterization of HCPT-MVLs

The particle size distribution of HCPT-MVLs was analyzed by laser-scattering particle size analyzer (Mastersizer 2000, Malvern, UK). The morphology was observed under a microscope (Axiovert 40 CFL, Carl Zeiss, Oberkochen, Germany), and the images were recorded photographically.

Fluorospectrophotometry analysis of HCPT

The reference standard of HCPT was dissolved in phosphate-buffered saline (PBS) (pH 12). HCPT-MVLs were processed as follows: 0.1 mL of HCPT-MVLs and 0.45 mL of chloroform were transferred to an eppendorf tube and vortexed for 3 minutes. Then 3 mL of PBS (pH 12) was added to the eppendorf tube and vortexed for another 4 minutes. The mixture was then centrifuged at $2500 \times g$ for 5 minutes, and the supernatant was diluted to appropriate concentration for determination (RF-5301 spectrofluorometer, Shimadzu Co. Ltd., Kyoto, Japan). Drug-free MVL was processed in the same way[26].

Table 1. Code and formulations of HCPT-MVLs.

Variables	Code	PC:Chol:SO	Weight ratios of lipid and HCPT phospholipid complex	Stirring time of primary emulsion (seconds)	Volume ratios of primary emulsion to aqueous solution (v/v)
MVL	A1	6:1:1	1:15	80	0.45
	A2	7:1:1	1:15	80	0.45
	A3	6:1:0.6	1:15	80	0.45
	B1	6:1:1	1:10	80	0.45
	B2	6:1:1	1:30	80	0.45
	C1	6:1:1	1:15	60	0.45
	C2	6:1:1	1:15	120	0.45
	D1	6:1:1	1:15	80	0.5
	D2	6:1:1	1:15	80	0.4

Determination of encapsulation efficiency

To determine the free drug concentration in the formulation, HCPT-MVL preparations (0.1 mL) were diluted to 2 mL with normal saline before determination. Resultant diluted suspension (1 mL) was filtered through a 0.22-μm cellulose acetate filter membrane to separate free HCPT and encapsulated HCPT. The filter membrane was rinsed with 1 mL of normal saline. The filtrate was collected together and adjusted with PBS (pH 12) to obtain the appropriate concentration for determination. The amount of free drug (D_{free}) and total drug amount (D_{tot}) were determined as described above. Encapsulation efficiency (EE%) of HCPT-MVLs was calculated by the following equation:

$$EE\% = \frac{D_{tot} - D_{free}}{D_{tot}}\%.$$

In vitro release studies

To investigate the in vitro release behaviors of HCPT from MVLs, a dialysis method was employed. HCPT-MVL suspension (0.5 mL) was placed in a dialysis bag (MW cutoff 8000–12,000), and the bag was introduced to the dissolution system and immerged in 800 mL of PBS (0.02 M, pH 7.4) containing 0.05% (w/v) of NaN_3. The PBS system was stirred at a speed of 25 rpm at 37°C. At predetermined time intervals, an aliquot of release medium was withdrawn from the system and analyzed by RF-5301 PC fluorospectrophotometry (Shimadzu), with a replenished equal amount of prewarmed fresh PBS. In addition, the in vitro release behavior of free HCPT was also measured as a control. The same amount of free HCPT was dissolved in PBS (pH 10). All the assays were performed in quadruplicate.

Pharmacokinetics in rats

Animals

Sprague–Dawley rats (female, 180–230 g) were purchased from the Experimental Animal Center of Sichuan University (protocol number for animal study: CSDGZ-10). The rats were housed at a room temperature of 22 ± 2°C and a relative humidity of 50 ± 10%. The animals were allowed ad libitum access to standard diet and water except wherever indicated. All the animals were used and treated as prescribed in the 'Guide for the Care and the Use of the Laboratory Animals' (NIH publication no. 92-93, revised 1985) and all the animal protocols and experiments were approved and supervised by Animal Ethics Committee of Sichuan University.

Drug administration and blood sampling

Two groups of rats ($n = 4$) were treated with HCPT-MVLs and HCPT solution at a single dose of 8 mg/kg through subcutaneous injection at the back, respectively. Blood samples (0.3 mL) were collected from the ophthalmic vein at predetermined time intervals after administration. The plasma was immediately separated by centrifugation at 5000 × *g* for 5 minutes. Rat plasma was obtained and stored at −20°C until analysis.

Sample preparation

Acetic acid (10 μL) was added to 100 μL of rat plasma to acidize the HCPT. After 2 hours away from light, 100 μL of mixed organic solvent of methanol and acetonitrile (1:1, v/v) were added to the mixture. The resulting mixture was vortexed for 5 minutes and then centrifuged at 10,000 × *g* for 10 minutes. Fifty microliters of the clear supernatant was injected into the high-performance liquid chromatography (HPLC) system for analysis.

High-performance liquid chromatographic analysis of HCPT

The in vivo concentration of HCPT was assayed on a Shimadzu LC-10AT (Shimadzu) HPLC system equipped with a fluorescence detector (RF-10AXL, Shimadzu) and a Scienhome C-18, 5 μm, 15 cm × 4.6 μm RP-HPLC analytical column. The mobile phase consisted of methanol/0.1 M ammonium acetate solution (adjusted to pH 6.0 with acetic acid) (50:50, v/v)[27]. The injection volume was 50 μL and eluted at a flow rate of 0.9 mL/min

at 35°C. The eluents were monitored with the highest fluorescence intensity at $\lambda_{ex}/\lambda_{em}$ = 378/550 nm.

Results and discussion

Influence of formulation and process parameters on particle appearance and encapsulation efficiency of HCPT-MVLs

To optimize the HCPT-MVLs' preparation, the influences of various process parameters were evaluated. The effects of weight ratios of PC:Chol:SO and HCPT:PC, the stirring time of original emulsion, and the volume ratio of original emulsion and secondary aqueous solution on particle appearance and encapsulation efficiency of HCPT-MVLs are shown in Figure 1. The appearance was divided into three grades (good, moderate, and bad) according to the size distribution, integrity, and morphology of partical size interior particles, and good, moderate, and bad were representative with 3, 2, and 1, respectively. The encapsulation efficiency of MVLs varied between 66.30% and 90.10%, depending on different process parameters. The highest encapsulation efficiency was obtained at a weight ratio of PC:SO:Chol prepared by 6:1:1 (quality ratios of PC:SO:Chol), a weight ratio of HCPT:PC of 1:15 (quality ratios of HCPT and lipid), continual stirring for 80 seconds (stirring time of original), and a ratio of primary emulsion to outer water phase of 0.5:1.

As a natural component of cell membrane, Chol was thought to have some effects on modulating the fluidity of the membrane. Therefore, Chol was often added in the formulation of liposomes to stabilize their structure. The weight ratio of PC:Chol in prior articles was usually about 8:5[17,19]. Different from such water-soluble drug formulation, HCPT–PCC, as a lipophilic complex, was supposed to locate on the phospholipid membrane, which would compete in occupation of lipid membrane with Chol. To increase drug-loading content, the amount of Chol used in our preparations was lower than that in the general formulation of MVLs. The encapsulation efficiency and stability of MVLs had direct relation to drug-to-phospholipid ratio. Usually, excessive drug went beyond the capacity of phospholipid; in that case, it was hard to get qualified and stable liposomes[28]. When the weight ratio of HCPT:PC was 1:10, MVL showed poor appearance and a relatively low encapsulation efficiency. However, better results could be obtained with the HCPT:PC weight ratio of 1:15 or

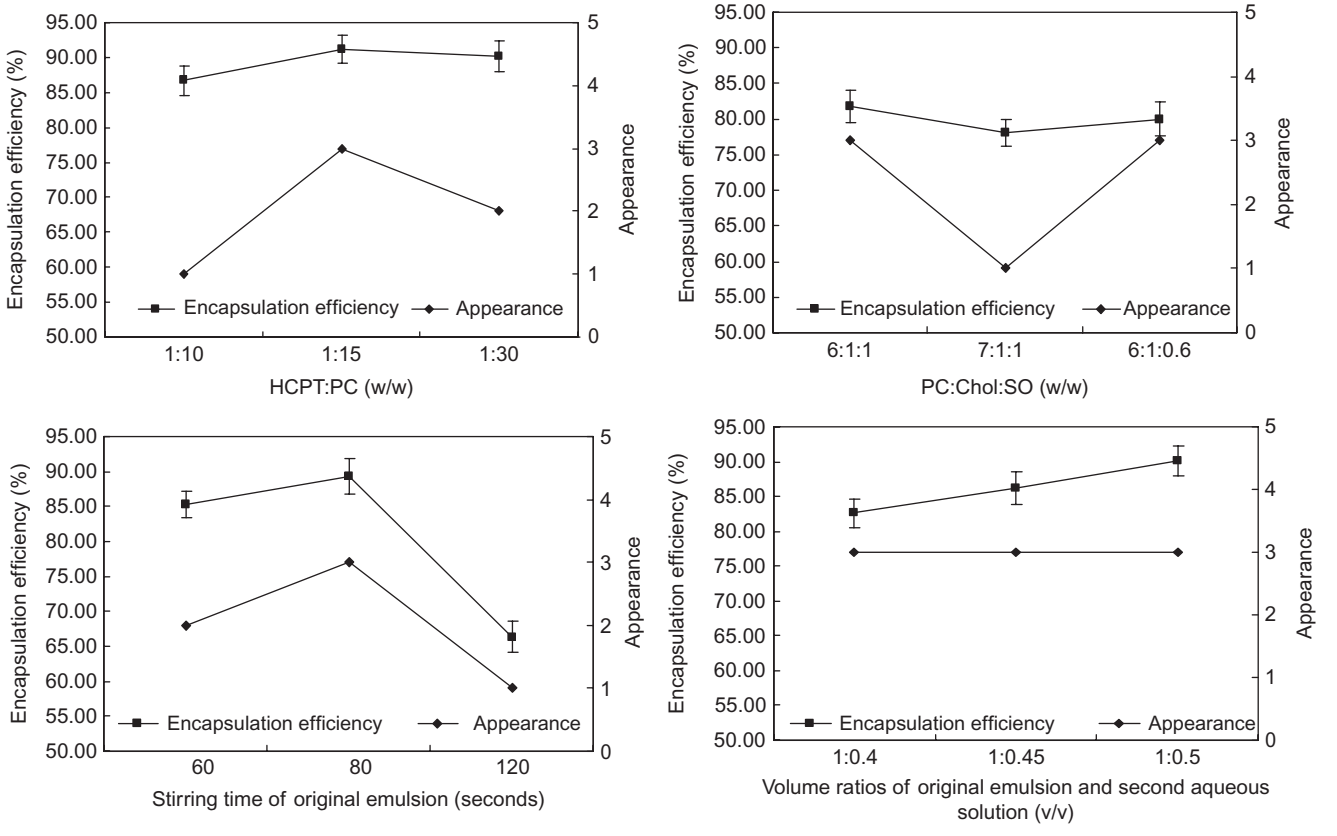

Figure 1. Effect of various process parameters on encapsulation efficiency and appearance of HCPT-MVLs. Data are means (n = 3).

1:30. Considering drug-loading content, the weight ratio of HCPT:PC of 1:15 was selected for further study. Oil phase was dissolved in mixed organic solvent. The organic solvent could be evaporated when original emulsion was subjected to high-speed stirring. Excessively long stirring time might lead to the excessive loss of water, which resulted in mastic-like appearance of primary emulsion. Inversely, if the stirring time was too short, the primary emulsion would be nonuniform. The volume ratio of primary emulsion and secondary aqueous solution had no effect on appearance but encapsulation efficiency. It could be inferred that proper ratio of primary emulsion and secondary aqueous solution was responsible for the formation of emulsion. The resultant emulsion was usually unstable when the phase volume fraction was lower than 20%, and the suitable phase volume fraction was between 20% and 50%[29].

Morphology and size of MVLs

The morphology of the MVLs was examined under microscope. As shown in Figure 2A, the MVLs exhibited spherical, honeycomb-like structure of tiny chambers. As seen in Figure 2B, a narrow and mono-modal distribution particles with a median size of 9 μm were prepared, and over 90% of the particles fell within a size range from 5 to 15 μm.

Encapsulation efficiency of HCPT-MVLs

Based on the fluorescence-scanning spectra, an excitation wavelength of 414 nm and an emission of 549 nm were employed for fluorospectrophotometry analysis. As shown in Figure 3, no interference peaks could be observed in the spectrum of blank MVL after sample processing courses. Also, no swift in emission peak was noted in HCPT-MVLs, so it can be concluded that no chemical changes or drug degradation occurred after HCPT was encapsulated into DepoFoam™.

The linear range of the standard curve was between 4 and 120 ng/mL with an R^2 of 0.9998. Recovery rate was 97.65%, 104.3%, and 99.9% for low, middle, and high concentration, respectively. Moreover, the interday and intraday relative standard deviations were all below 3.5%.

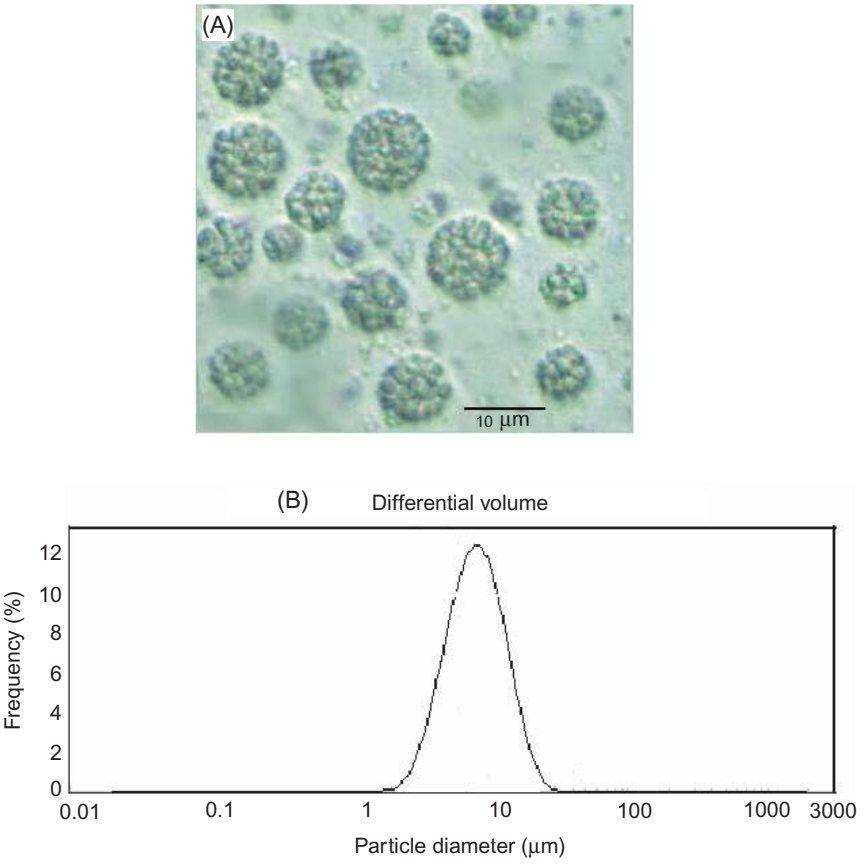

Figure 2. (A) Microphotograph of HCPT-MVLs at × 400 magnification; (B) size distribution of HCPT-MVLs measured by laser-scattering method.

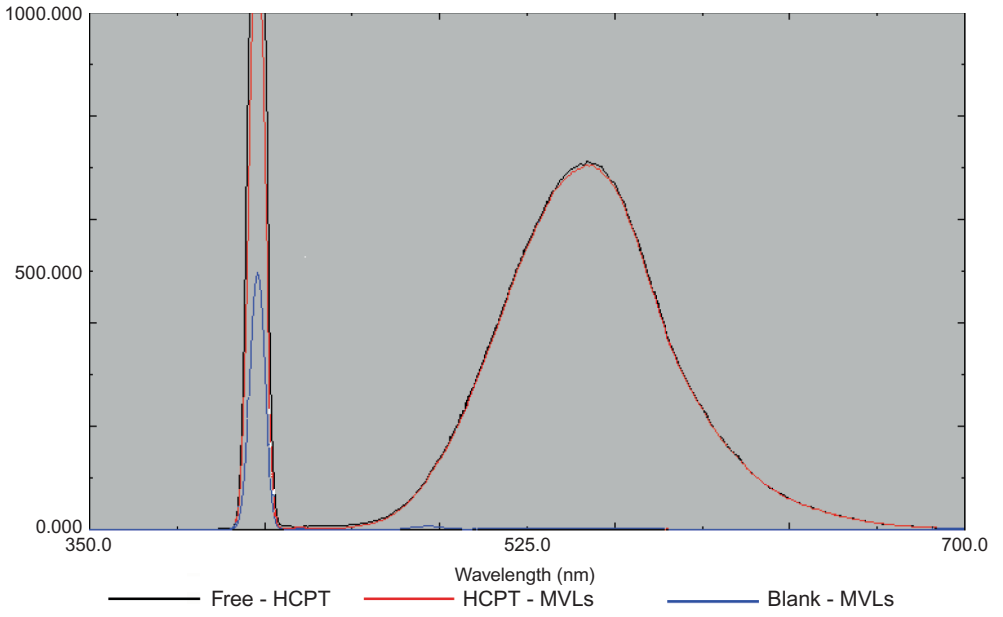

Figure 3. Fluorescent scanning of free HCPT, processed HCPT-MVLs, and blank MVLs.

HCPT shows intensive fluorescence, and adjuvants or excipients of HCPT-MVLs have no absorbance after sample processing. Therefore, fluorospectrophotometry was selected to measure encapsulation efficiency and in vitro release of HCPT-MVLs for its simplicity and high sensitivity. The intensity of fluorescence absorption is different between carboxylate and lactone form, so the sample was alkalized with PBS (pH 12) to obtain simple carboxylate form of HCPT.

The high encapsulation efficiency may be attributed to the increased lipophilicity of HCPT–PCC and the unique structure of MVLs. The HCPT–PCC showed good lipid affinity that sticked to the lipid domain of the phospholipid membrane. It is different from water-soluble drug that locates to the inner aqueous phase. Structural studies on DepoFoam particles using transmission electron microscopy[16] and confocal microscopy utilizing fluorescence-labeled lipids[8] indicated that there are large areas of phospholipid membrane between the internal chambers.

In vitro release

Figure 4 shows the morphology of HCPT-MVLs before and after release in PBS for 6 days. As shown in the figure, by day 6, particles shrinked obviously and most of the particles were degraded. There were only a few honeycomb-like particles in the visual field. The outmost membranes of an MVL resulted in the release of encapsulated drug to the external medium and the particle

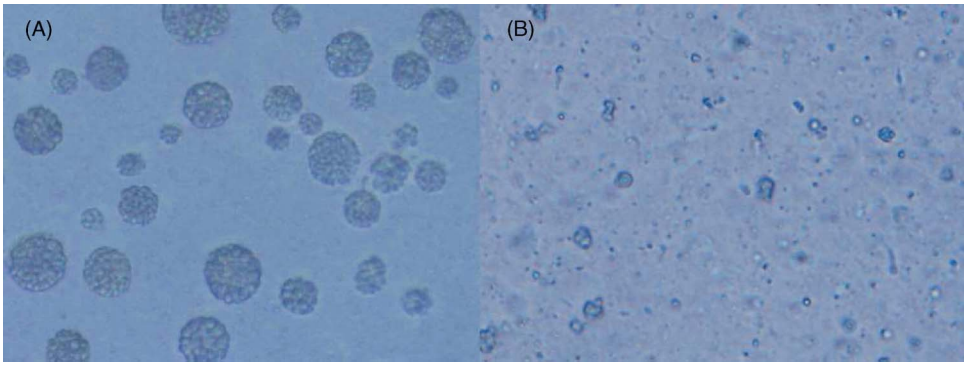

Figure 4. Light microscopy images (40 × 10) of HCPT-MVLs before (A) and after (B) drug release in PBS at 37°C under dynamic conditions (25 rotation cycles/min).

size decreased with the breach of external membrane. The mechanism of release may be composed of at least three components including diffusion, erosion, and attrition[30].

The in vitro drug release behavior of HCPT-MVLs was evaluated and compared to that of HCPT solution in Figure 5. In stark contrast to free drug, sustained release was observed for HCPT-MVLs. Fifty percent of the loaded drug was released after 50 hours, and 80% of the drug was released in 140 hours approximately. In comparison, the release of free HCPT was rather rapid, with an accumulated release amount of 90% within 7.5 hours. These data confirmed the sustained-release pattern of HCPT-MVLs, which suggested that prolonged in vivo release of HCPT was highly possible.

The extended release can be attributed to the depot function of DepoFoam™ technology. MVLs have a larger size and volume than conventional liposomes, which are composed of nonconcentric multiple lipid layers. Therefore, the drug is mainly encapsulated in the internal layers. Moreover, the interconnected network of the MVL structure also ensures that the vesicles can rearrange themselves internally without release of drug by internal fusion and division. Hence, by incorporation into MVLs, the stability and sustained release of HCPT could be obtained. Furthermore, the sustained-release behavior might be partially ascribed to the strong interactions between drug and phospholipd of HCPT-PC.

In vivo study

Validation of method

Analytical method was validated according to the following criteria: linearity, precision (within- and between-day variability), limits of detection and quantification, and recovery and stability (room temperature and –20°C). The calibration curves were plotted in the range of 2–229 ng/mL, with a correlation coefficient of 0.9972. Interday and intraday relative standard deviations were all below 4% with a satisfactory recovery. The limit of detection was 0.5 ng/mL and limit of quantification was 2.0 ng/mL. The sample was stable when stored at room temperature for 6 hours and –20°C for 48 hours.

Pharmacokinetic study

Figure 6 showed drug concentration–time curves after a single subcutaneous administration of HCPT-MVLs and free drug (8 mg/kg) into rats. The pharmacokinetic parameters were analyzed using DAS (drug and statistics) computer program and also listed in Table 2.

As shown by plasma drug concentration–time plot (Figure 6), significant prolonged drug release of HCPT from MVLs could be observed, compared with free HCPT. Initially, the concentration of free HCPT in plasma was extremely high after the administration of solution and decreased rapidly afterwards. In contrast, the HCPT concentration curve obtained by MVLs maintained steady with negligible initial burst release. Meanwhile, the drug was detected over a period of 6 days, which is in concert to in vitro release study.

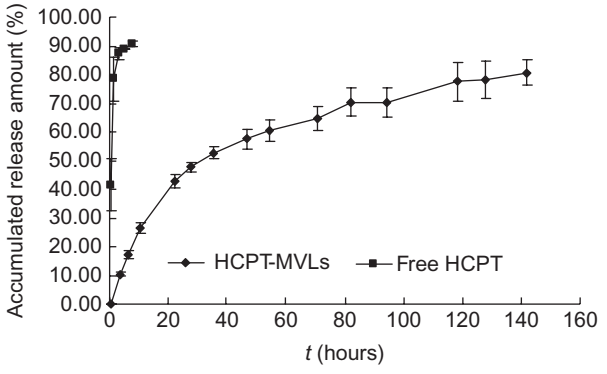

Figure 5. In vitro release profile of HCPT from HCPT-MVLs (Samples were suspended in phosphate-buffered saline, pH 7.4, at 37°C versus free HCPT in the medium. $n = 4$).

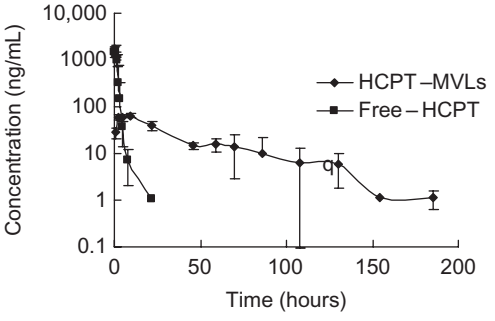

Figure 6. Pharmacokinetic profile in rats after receiving a single subcutaneous administration of HCPT-MVLs and HCPT solution (8 mg/kg) ($n = 4$).

Table 2. Pharmacokinetic parameters of HCPT-MVLs and HCPT solution in rats after subcutaneous injection (8 mg/kg) ($n = 4$).

Formulation	$AUC_{(0-t)}$ (ng·h/mL)	$AUC_{(0-\infty)}$ (ng·h/mL)	$MRT_{(0-t)}$ (hours)	$MRT_{(0-\infty)}$ (hours)	$t_{1/2}$ (hours)	C_{max} (ng/mL)
MVLs	2797.57 ± 909.36	2943.99 ± 787.77	40.01 ± 7.01	51.65 ± 13.20	30.94 ± 13.20	60.88 ± 9.77
Solution	3350.94 ± 1008.92	3360.61 ± 1023.29	1.71 ± 0.17	1.73 ± 0.16	0.98 ± 0.23	1819.17 ± 406.40

AUC, the area under the concentration–time curve; MRT, mean residence time; $t_{1/2}$, half-life; C_{max}, the maximum concentration.

As shown in Table 2. The data indicated that the pharmacokinetic parameters of HCPT-MVLs were distinct from HCPT solution. The HCPT-MVLs were shown to possess sustained-release characteristics as evidenced by the increase in $t_{1/2}$ for 0.983–30.94 hours. The $MRT_{(0-t)}$ and $MRT_{(0-\infty)}$ of the HCPT-MVLs were significantly prolonged when compared with HCPT solution from 1.708 to 40.005 hours and 1.725 to 51.652 hours, respectively. The maximum plasma concentration of HCPT-MVLs was only 60.877 ng/mL, which was 1/300 to HCPT solution approximately. The changes have been achieved without significant decrease in the $AUC_{(0-t)}$ and $AUC_{(0-\infty)}$ of the HCPT-MVLs. This slow release pattern can be explained by the formation of a lipid reservoir at the injection site from which the drug is slowly released into the tissue fluids[31–33]. These results indicated that sustained-release effect and no dramatic decrease in the bioavailability of HCPT-MVLs could be obtained. We can conclude that DepoFoam™ technology not only suits for soluble drugs to attain sustained release but also serves as a useful strategy for insoluble drugs. Furthermore, HCPT solution was expected to be absorbed rapidly as there were substantial changes in plasma concentration, which might increase the systemic toxicity. The stable and low concentration of MVLs was relatively safer, and it was easier for clinical application.

MVLs are biodegradable, biocompatible[31], easy to produce, and can be used for intrathecal, subcutaneous, intramuscular, intraperitoneal, and intraocular administration[34]. Different from pure phospholipids such as dioleoylphosphatidylcholine and dipalmitoylphosphatidylglycerol (DPPG), which were used in most other literatures, the negative charge of DPPG can keep MVLs stable by electrostatic repulsion. The price of dioleoylphosphatidylcholine and DPPG is expensive; E-80 was used to lower production cost. and Myrj 59 was used to stabilize the MVLs in our study. The effectiveness of Myrj 59 in stabilizing the MVLs presumably stem from its dual hydrophobic–hydrophilic nature, whereas the extensive hydrophilic PEG chain head group extends into water and sterically hinders the particles from approaching each other[35]. As a series nonionic emulsifier, polyoxyethylene stearate was used in some studies[36–39], although it has not been approved to parenteral delivery. It would be further studied about emulsifier compatibility, but it provided a clue that we could find another approved emulsifier with similar structure such as Solutol HS 15.

In view of formulations approved by the US FDA for clinical use, three directions to stabilize the HCPT-MVLs could be done. First, the MVLs should be stored at 2–8°C and should not freeze. Second, aggressive shaking should be avoided. Third, the headspace full of inert gas such as nitrogen should be kept.

Conclusions

This study demonstrated the possibility of applying DepoFoam™ technology for HCPT. MVL delivery systems for HCPT with high encapsulation efficiency, excellent appearance, and particle size were prepared by double emulsification process. Meanwhile, HCPT-MVLs exhibited satisfactory sustained-release effect in both in vitro and in vivo studies. Therefore, this study provides an attempt and a reference for sustained release of insoluble drugs by coupling of DepoFoam™ and phospholipid complex techniques.

Acknowledgments

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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