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Original Article

Therapeutic effect of folic acid modified hydroxycamptothecine nanoliposomes for hepatoma SMMC-7721 cells both *in vitro* and *in vivo*

Yuan Tian[1*], Xiao Hu[2*], Liangping Li[2], Xun Xiao[2]

[1]Department of Digestive Endoscopy, Guizhou Provincial People's Hospital, 83 East Zhongshan Rd., Guiyang 550002, Guizhou, P. R. China; [2]Department of Gastroenterology, Sichuan Academy of Medical Sciences and Sichuan Provincial People's Hospital, No. 32, Xierduan, Chengdu 610072, Sichuan Province, P. R. China. [*]Equal contributors and co-first authors.

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Abstract: Objective: This study aims to prepare folic acid (FA) modified hydroxycamptothecine (HCPT) nanoliposomes (FA-HCPT-LP NPs) and investigate their targeting property and therapeutic effect for hepatoma SMMC-7721 cells both *in vitro* and *in vivo*. Method: Film-dispersion method was used to prepare FA-HCPT-LP NPs. A nanosizer was used to detect the size and zeta potential of FA-HCPT-LP NPs. A UV spectrophotometer determined the encapsulation efficiency and release rate of HCPT. The half-life in mice blood and content in tumor and major organs of FA-HCPT-LP NPs were characterized by radioactive ^{125}I labeling. The uptake of FA-HCPT-LP NPs in hepatoma cells was observed under confocal microscopy. CCK-8 method detected the effect of FA-HCPT-LP NPs on cell viability and the inhibitory effect of FA-HCPT-LP NPs on subcutaneously transplanted tumor. Results: The size distribution of FA-HCPT-LP NPs ranged from 110 to 155 nm, averaging 128 nm, and the zeta potential was -31.9 mV. When mass ratio of HCPT to soyabean lecithinhe was 1:10, the maximum encapsulation efficiency of HCPT was 71.5±2.6%. Lyophilized FA-HCPT-LP NPs were more stable than liquid ones. The maximum releasing ratio of HCPT was 61.9±3.1% under 37°C. FA-HCPT-LP NPs could be efficiently uptaken by SMMC-7721 cells and could largely accumulate in tumor tissues directionally, inhibiting the viability of hepatoma cells and the growth of subcutaneously transplanted tumors effectively. Moreover, FA-HCPT-LP NPs had longer half-life than free HCPT in blood. Conclusion: In this study, we successfully prepared tumor-targeting FA-HCPT-LP NPs that could be uptaken by hepatoma cells with high efficiency and target tumor cells with good anti-cancer effect both in vitro and in vivo.

Keywords: Folic acid, hydroxycamptothecine (HCPT), nanoliposome, targeted therapy

Introduction

Camptothecin (CPT) was a kind of alkaloid in camptotheca acuminate, which was a deciduous plant belonging to camptotheca (nyssaceae) [1]. In 1950, Wall found that hydroxycamptothecin (HCPT) was the most active indole alkaloid among natural or synthesized CPT analogues [2, 3]. Studies found that HCPT was of good effect for such malignant tumors as adenogenous epithelioma of the head and neck, chronic granulocytic leukemia (CGL) and rectal cancer [4-6]. Currently, HCPT in clinical use included sodium injection, sodium powder and capsule [7, 8]. However, these dosage forms had their defects, respectively. With a phenolic hydroxyl group in the molecular structure of HCPT, sodium injection was unstable in the presence of light or heat or when it was exposed in the air. HCPT was ready to be oxidized and hydrolyzed so that its content decreased. At the same time, the lactone ring of HCTP would be opened. Thus, the effect was decreased. In addition, the half-life of HCPT was short in blood. Compared with sodium injection, sodium powder was more stable. Nevertheless, its half-life was still short. Oral administration of capsules was applicable for the treatment of gastroenteric tumor. But increased dosages raised its toxic and side effects. Therefore, it was of great significance for the clinical use of HCPT to find out a new dosage form which could improve its half-life in blood and its efficacy.

Liposomes, as natural self-enclosed solid utricles, were new passive targeting preparations,

which had such advantages as bio-degradation and no toxicity or immunogenicity [9]. In recent years, it was found that using liposomes as carriers could not only decrease dosage and toxic and side effects, but also prolong the half-life in blood. In this study, nanoliposomes were used as carriers to encapsulate HCPT. Furthermore, the surface of these liposomes was modified with folic acid (FA), obtaining HCPT nanoliposomes with targeting property.

Materials and methods

Major reagents

HCPT powder (> 98%) was purchased from Xi'an Realin Biotechnology Co., Ltd.; soyabean lecithin, cholesterol and α-tocopheryl from Sinopharm Chemical Reagent Co., Ltd.; DSPE-PEG_{2000}-FA, Rhodamine (Rho), 4',6-diamidino-2-phenylindole (DAPI), $Na^{125}I$ and chloramine-T from Sigma (the US); RPMI-1640 culture solution, fetal calf serum, trypsin-EDTA digestive juices, phosphate buffer from Hylcon (the US); and Cell Counting Kit (CCK-8) from Dojindo (Nippon).

Major instruments

The nanosizer (ZEA3600) was purchased from Malvern Instruments Ltd; UV spectrophotometer (UV-2550) from Shimadzu Corporation (Nippon); confocal microscope (FV1200) from OLYMPUS; microplate reader (SpectraMax M5) from Molecular Devices (the US); and CO_2 incubator (MDF-U71V) from SANYO (Nippon).

Cell line and laboratory animals

Human hepatoma cell line SMMC-7721 was purchased from the cell bank of China Committee for Typical Culture Collection, China Academy of Sciences. Laboratory mouse 22-25 g aged from 5 to 6 weeks balb/c (nude mouse) was purchased from Hunan SJA Laboratory Animal Co., Ltd. (production license No: SCXY (Xiang) 2011-0003).

Experimental methods

The preparation of FA-HCPT-LP NPs: Film dispersion method was used to prepare FA-HCPT-LP NPs in this study. A prescribed dose of soyabean lecithin, cholesterol, α-tocopherol and DSPE-PEG_{2000}-FA was dissolved by ethanol-sonication method, with the mass ratio of soyabean lecithin to cholesterol 2:1. The dissolved matter was placed in a round-bottom flask and several steel balls were added. Then, the flask received rotary evaporation under 40°C until no ethanol was left, when a layer of homogeneous emulsus film was formed. Then, rotary evaporation continued until the film became dry enough. Afterwards, a certain amount of PBS buffer (pH 7.4) was added into the flask. The resultant mixture was put in a table concentrator, concentrated at 200 rpm under 60°C, and hydrated for 50 min. After being placed at room temperature, it was homogenized with a high speed homogenizer for 20 min. Finally, it was filtered with a 0.45 and 0.22 μm millipore film for three times, respectively and FA-HCPT-LP NPs was obtained.

The characterization of FA-HCPT-LP NPs: A proper amount of FA-HCPT-LP NPs was detected with a nanosizer for their size and zeta potential and the content of HCPT was detected by UV spectrophotometer at 266 nm. The encapsulation efficiency of HCPT EE% = $W_{Encapsulation}/W_{fed} \times 100\%$, in which $W_{Encapsulation}$ was the amount of HCPT encapsulated in liposomes and W_{fed} was the amount of HCPT fed in total. Besides, changes of encapsulation efficiency over time were detected for HCPT solution and lyophilized HCPT.

Cell culture and activity test: Human hepatoma cell line SMMC-7721 was cultured in RPMI-1640 culture solution with 10% fetal calf serum and 1% double-antibody, which was placed in a constant incubator with 5% carbon dioxide at 37°C. Cells during the logarithmic phase were inoculated in a 96-well plate (100 μl cell suspension/well, 10^5 cells/ml). When cell attachment was observed, samples with different concentrations were added and cultured for different periods of time. Then, the old culture solution was removed and a new one with 10% CCK-8 working solution was added. After incubating for 30 minutes, the absorbance value at 450 nm (OD450 nm) was detected. The bigger OD450 nm was, the higher cell activity was.

The targeting property and pharmacokinetics of nanoliposomes: In this study, Rhodamine was used as a molecular marker for nanoliposomes. FA-HCPT-LP NPs and HCPT-LP NPs labeled by Rhodamine were incubated with hepatoma cells for 3 hours, respectively. Then,

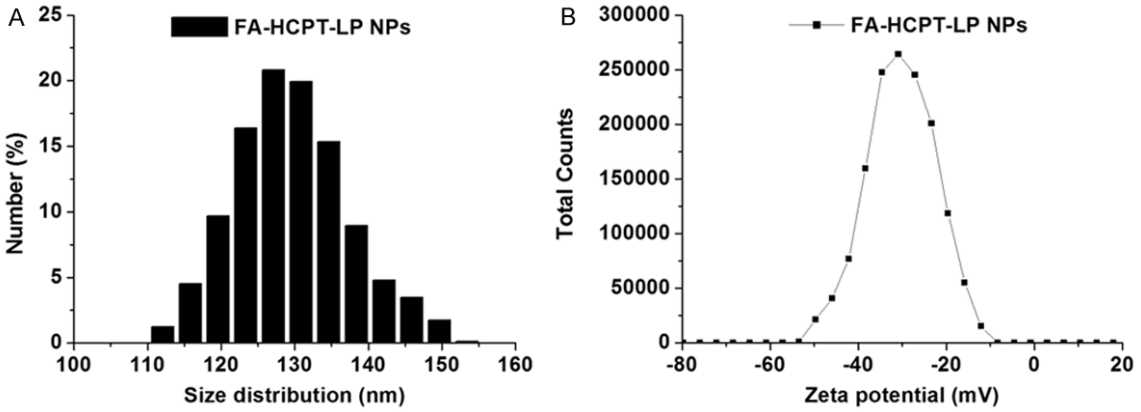

Figure 1. The size distribution of FA-HCPT-LP NPs (A) and zeta potential distribution of FA-HCPT-LP NPs (B).

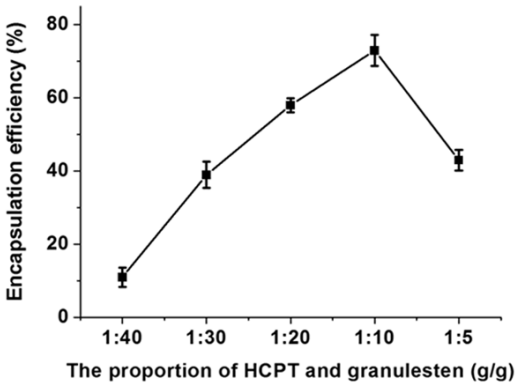

Figure 2. The encapsulation efficiency of HCPT in FA-HCPT-LP NPs when the proportion of HCPT and granulesten changed from 1:40 to 1:5.

these mixtures were washed by PBS for three times. Afterwards, nucleus-specific dye DAPI was added and incubated with cells for 20 minutes. Again, it was washed by PBS for three times and observed under confocal microscopy for the fluorescence signal of Rhodamine and DAPI in cells. In addition, chloramine-T method (by the oxidation of ^{125}I-labeled substance) was used to label nanoliposomes as follows: 10 μl sample, 300 μl Ci Na^{125}I and 100 μl chloramine-T (2 mg/ml) were mixed at room temperature (pH 7.5) for reaction for an hour; and then, redundant ^{125}I was dialyzed until no γ-ray activity of ^{125}I was left. Labeled samples were stored in a dark place at low temperature.

The content of FA-HCPT-LP NPs in tumor-bearing nude mouse was detected as follows: 24 tumor-bearing nude mouse (half male and half female) were weighed and randomized into two groups-FA-HCPT-LP NPs and HCPT-LP NPs (12 mouse/group); In each group, FA-HCPT-LP NPs and HCPT-LP NPs labeled by 37MBq^{125}I were infused through caudal vein, respectively; 24 hours after administration, these mouse were killed and their heart, live, spleen, lung, kidney and tumor sphere were taken out; After being rinsed, they were dried out with filter paper and put into a pre-weighed PVC test tube to weigh them; Besides, the specific radioactivity (cpm/g) of each tissue was measured and calculated and their ID%/g value (the percentage of dose uptaken by each gram of tissue) was also calculated. The calculation formula of ID%/g was as follows:

The blood half-life was detected as follows: 9 tumor-bearing nude mouse (half male and half female) were randomized into three groups-HCPT, FA-HCPT-LP NPs and HCPT-LP NPs (3 mouse/group); HCPT, FA-HCPT-LP NPs and HCPT-LP NPs labeled by 37MBq^{125}I were infused through caudal vein; Venous blood was collected from their ear vein 0 h, 0.5 h, 1 h, 2 h, 3 h, 6 h, 12 h, 24 h, 36 h and 48 h after administration. The specific radioactivity (cpm/g) was measured and used for pharmacokinetic analysis.

The establishment of tumor model and treatment: The animal model with subcutaneously transplanted tumors was established in the following steps: PBS solution with 150 μl 10^6 SMMC-7721 cells was injected at the left back of mouse; The tumor size was observed every day until it reached up to about 300 mm^3 when the tumor-bearing nude mouse were randomized into four groups with normal saline (con-

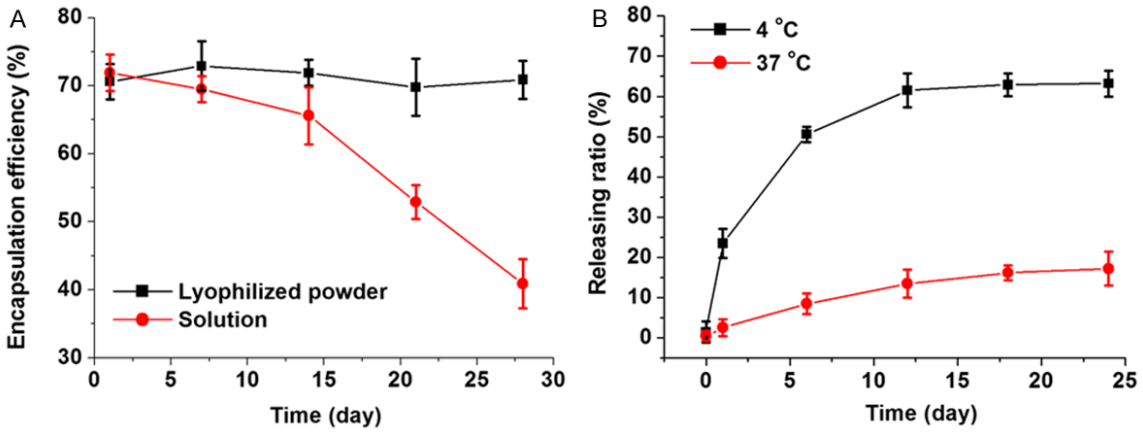

Figure 3. The stability of FA-HCPT-LP NPs (A) and the releasing ratio of HCPT at 4 °C and 37 °C (B).

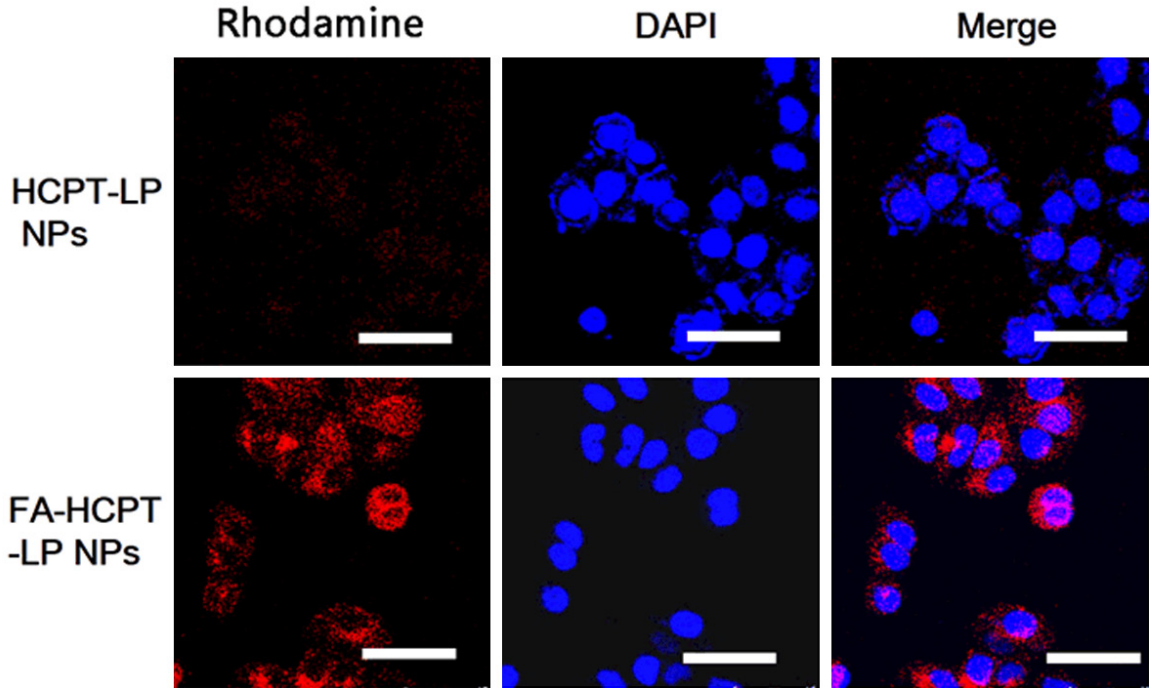

Figure 4. The cell uptake of FA-HCPT-LP NPs, Column 1: Rhodamine channels showing the red fluorescence from Rhodamine labeled liposomes distributed in cytoplasm. Column 2: DAPI channels showing the blue fluorescence from DAPI stained nuclei. Column 3: Merged channels of Rhodamine and DAPI channels. Scale bar = 50 nm.

trol), HCPT, HCPT-LP NPs and FA-HCPT-LP NPs (7 mouse/group). The tumor size was measured every four days.

Statistical analysis

Data in this study was expressed as $\bar{x} \pm$s.d. and statistical analysis was performed by using software SPSS17.0. Comparison between two groups was conducted by using independent sample t-test. *P < 0.05 meant that there was statistically significant difference. **P < 0.01 meant that there was extremely statistically significant difference.

Results

The characterization of FA-HCPT-LP NPs

The detection result obtained by using a nanosizer was shown in **Figure 1A**. The size distribution of FA-HCPT-LP NPs ranged from 110 to

155 nm, averaging 128 nm. So it belonged to nano-scale materials. As shown in **Figure 1B**, the zeta potential of FA-HCPT-LP NPs was within -60 mV--10 mV, averaging -31.9 mV, indicating that FA-HCPT-LP NPs was of good water solubility. **Figure 2** suggested the impact of feed ratio of HCPT to soyabean lecithin on HCPT encapsulation efficiency during the preparation of nanoliposomes. It was found that when the ration was 1:10, the encapsulation efficiency of HCPT was the highest, namely, 71.5±2.6%. In 28 days, the encapsulation efficiency of lyophilized FA-HCPT-LP NPs and FA-HCPT-LP NPs liquid was detected every week and results (see **Figure 3A**) indicated that the former was more stable than the latter. Furthermore, the release rate of HCPT was detected under 4°C and 37°C, respectively. As shown in **Figure 3B**, the maximum releasing ratio of HCPT under 37°C was 61.9±3.1%, suggesting susceptibility to temperature.

The cell uptake of FA-HCPT-LP NPs

The cell uptake results for HCPT-LP NPs and FA-HCPT-LP NPs observed under laser confocal fluorescence microscope were presented in **Figure 4**. The Rhodamine fluorescence signal intensity was strong in the cytoplasm treated by FA-modified HCPT-LP NPs, while weak in that of HCPT-LP NPs. It suggested that FA obviously enabled nanoliposomes to target cell surface and thus be uptaken by cells.

The cytotoxicity test of FA-HCPT-LP NPs

As shown in **Figure 5A**, no evident impact on cell viability was found after cells being treated with 0-100 μg/ml liposomes for 24 h. It indicated that blank liposome carriers used in this study had no toxic or side effects at a certain concentration. **Figure 5B** showed that HCPT alone had a certain killing effect, while the cytotoxicity of FA-HCPT-LP NPs was more significant and concentration-dependent. It suggested that the targeting property of FA could increase the toxicity of HCPT for cells.

The blood half-life and tissue assay of FA-HCPT-LP NPs

As shown in **Figure 6A**, 48 h after HCPT, HCPT-LP NPs and FA-HCPT-LP NPs were injected through caudal vein, the blood half-life of HCPT alone was 2.1±2.9 h, while that of HCPT-LP NPs and FA-HCPT-LP NPs was about 20.4±1.9 h. Obviously, the latter was higher than the former, indicating that liposomes significantly increased the blood half-life of HCPT. Moreover, the modification of FA had no evident impact on the half-life of liposomes. 24 h after HCPT-LP NPs and FA-HCPT-LP NPs were injected through caudal vein; the content distribution in major organs and tumor regions was shown in **Figure 6B**. It was found that in addition to liver and kidney with vigorous metabolism, the content of HCPT-LP NPs and FA-HCPT-LP NPs in the heart, spleen and lung was very low. Besides, the content of FA-HCPT-LP NPs in tumor regions was evidently higher than that of HCPT-LP NPs ($P < 0.01$). It suggested that the targeting property of FA was very good and thus drugs could be transmitted to tumor regions actively.

The in vivo therapy of FA-HCPT-LP NPs

As shown in **Figure 7**, the normal saline control and HCPT alone had no significant impact on tumor growth. HCPT-LP NPs had a certain inhibitory effect only for the first 8 days after injection. After that, the tumor continued to grow. By contrary, FA-HCPT-LP NPs inhibited the growth of tumors until they disappeared.

Discussion

HCPT was a derivative of camptothecin. Studies found that its activity was stronger than camptothecin [10, 11]. Due to its poor water solubility, in clinical use, it was prepared into injection solution by opening lactonic ring to form sodium salt [12]. The lactone structure was essential for camptothecin derivatives to act on target enzymes. Therefore, the renal toxicity of HCPT injection solution increased and its activity decreased significantly [13]. Furthermore, it was found that the blood half-life of HCPT was extremely short [14].

In this study, liposomes were used to envelope HCPT. In this way, HCPT nanoliposomes were obtained. Liposomes, with a lipid bilayer, were characterized by such features as passive targeting property, long circulation, low toxic and side effect, as well as very good biocompatibility. They could improve the biocompatibility and blood half-life of HCPT. As shown in **Figure 6A**, the blood half-life of HCPT nanoliposomes was evidently longer than that of HCPT alone. Hence, liposomes, as carriers, made up for the

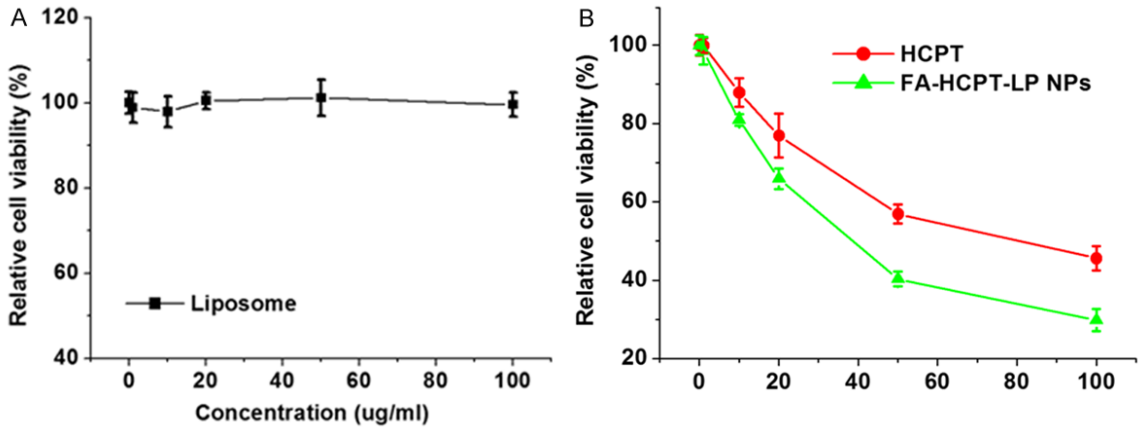

Figure 5. The cytotoxicity of blank liposomes without HCPT loaded (A) and the cytotoxicity of HCPT and FA-HCPT-LP NPs when the concentration of HCPT from 0 to 100 μg/ml (B).

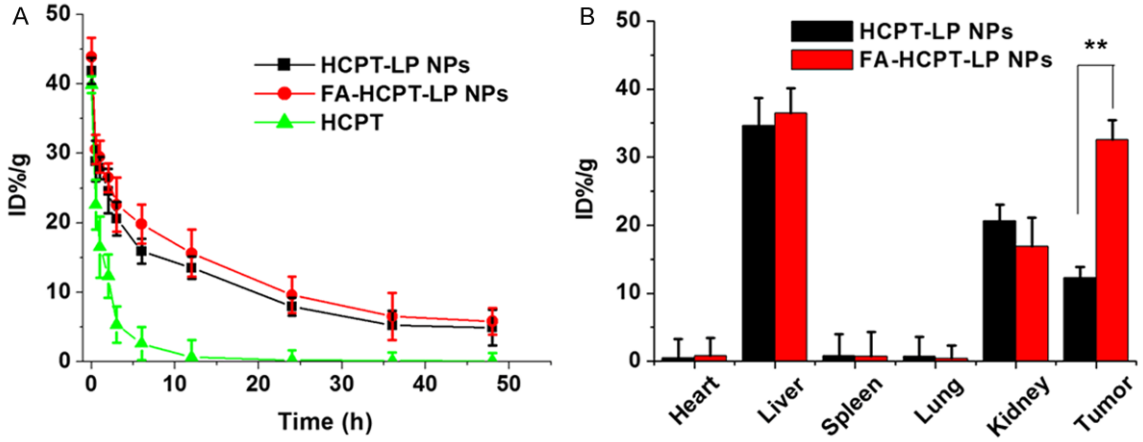

Figure 6. The blood half-life of HCPT, HCPT-LP NPs and FA-HCPT-LP NPs in 48 h (A) and tissue distribution of HCPT-LP NPs and FA-HCPT-LP NPs (B), $^{**}P < 0.01$.

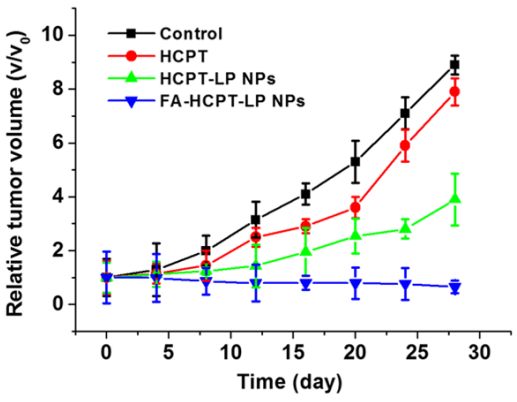

Figure 7. In vivo anti-tumor effect of different HCPT formulations in tumor-bearing mice.

poor biocompatibility and short half-life of HCPT.

However, as a therapeutic drug for cancer, HCPT nanoliposomes could not give full play to its anti-cancer effect with passive targeting property alone. Therefore, the surface of liposomes needs to be modified by a specific active targeting molecule based on the characteristics of tumor cells. It was reported that the expression of FA receptors was high on the surface of hepatoma SMMC-7721 cells, but low on the surface of normal tissue cells [15]. Thus, we modified the surface of HCPT liposomes by FA, the specific donor of FA receptors, and obtained FA-modified HCPT nanoliposomes. Results of *in-vitro* cell uptake test showed that FA-modified liposomes could be uptaken into cytoplasm with high efficiency, while only a few ordinary liposomes could be uptaken. It indicated that FA had a highly efficient targeting property. Results of cytotoxicity test suggested

that HCPT liposomes modified by FA had better cell killing effect. It demonstrated the highly efficient active targeting property of FA from another perspective. In addition, the content of FA-modified liposomes was significantly higher than that of HCPT nanoliposomes in tumor spheres. It further confirmed the highly efficient targeting property of FA. Moreover, both *in-vitro* and *in-vivo* test showed that FA-modified HCPT nanolipsomes had better anti-tumor effect compared with HCPT nanolipsomes alone.

In conclusion, we prepared FA-modified HCPT nanoliposomes with good biocompatibility and high encapsulation efficiency in this study. It was demonstrated in *in-vitro* and *in-vivo* test that they had highly efficient targeting property and therapeutic effect for hepatoma SMMC-7721 cells.

Disclosure of conflict of interest

None.

Address correspondence to: Xun Xiao, Department of Gastroenterology, Sichuan Academy of Medical Sciences and Sichuan Provincial People's Hospital, No. 32, Xierduan, Chengdu 610072, Sichuan Province, P. R. China. E-mail: xiaoxsccd@sina.com

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