ORIGINAL ARTICLE

Synergetic delivery of triptolide and Ce6 with light-activatable liposomes for efficient hepatocellular carcinoma therapy

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KEY WORDS
Hepatocellular carcinoma; Synergetic delivery; Triptolide; Ce6; Photo-activatable liposomes;

Abstract Hepatocellular carcinoma (HCC) has been known as the second common leading cancer worldwide, as it responds poorly to both chemotherapy and medication. Triptolide (TP), a diterpenoid triepoxide, is a promising treatment agent for its effective anticancer effect on multiple cancers including HCC. However, its clinical application has been limited owing to its severe systemic toxicities, low solubility, and fast elimination in the body. Therefore, to overcome the above obstacles, photo-activatable liposomes (LP) integrated with both photosensitizer Ce6 and chemotherapeutic drug TP (TP/Ce6-LP) was designed in the pursuit of controlled drug release and synergetic photodynamic therapy in HCC.

Abbreviations: ALT, liver-related alanine aminotransferase; AST, aspartate aminotransferase; BCA, bicinchoninic acid; BUN, blood urea nitrogen; Chol, cholesterol; CK, creatine kinase; CK-MB, creatine kinase-MB; CLSM, confocal laser scanning microscopy; Cr, creatinine; DEE, drug encapsulation efficiency; DLS, dynamic light scattering; Dox, doxorubicin; DSPG, distearoyl phosphatidylglycerole; EPR, enhanced permeability and retention; FBS, fetal bovine serum; FCM, flow cytometry; HCC, hepatocellular carcinoma; LDH, lactate dehydrogenase; LP, liposomes; NIR, near-infrared; PDT, photodynamic therapy; PDX, patient-derived xenograft; PDXHCC, patient derived tumor xenograft of HCC; Pt, platinum; ROS, reactive oxygen species; So, sorafenib; TEM, transmission electron microscope; TP, triptolide; TP/Ce6-LP, liposomes integrated with both photosensitizer Ce6 and chemotherapeutic drug TP; TUNEL, dT-mediated dUTP Nick-End Labeling.

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therapy. The TP encapsulated in liposomes accumulated to the tumor site due to the enhanced permeability and retention (EPR) effect. Under laser irradiation, the photosensitizer Ce6 generated reactive oxygen species (ROS) and further oxidized the unsaturated phospholipids. In this way, the liposomes were destroyed to release TP. TP/Ce6-LP with NIR laser irradiation (TP/Ce6-LP + L) showed the best anti-tumor effect both in vitro and in vivo on a patient derived tumor xenograft of HCC (PDXHCC). TP/Ce6-LP significantly reduced the side effects of TP. Furthermore, TP/Ce6-LP + L induced apoptosis through a caspase-3/PARP signaling pathway. Overall, TP/Ce6-LP + L is a novel potential treatment option in halting HCC progression with attenuated toxicity.

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1. Introduction

Hepatocellular carcinoma (HCC) occurs after long-term liver disease, and has become the most prevalent form of primary liver cancer\(^1\). HCC constitutes the second largest cause of cancer mortality in many countries\(^2\). Thus, it is one of massive global health problems today\(^3\). Up to now, in order to cure HCC, many strategies have been extensively employed, such as hepatic resection, orthotopic liver transplantation, chemoembolization, transarterial embolization and biotherapies\(^4,5\). However, the survival rate of HCC patient remains poor till far, due to the limitations of present strategies\(^6\). For example, less than 30% of patients are reported to receive specific interventions after liver transplantation, surgery, or chemoembolization\(^7\). Moreover, systemic chemotherapies lack target selectivity and are typically accompanied with multi-drug resistance as well as other severe side effects\(^8\)\(^9\)\(^10\). Therefore, the dismal prognosis for most HCC patients emphasizes a pressing need for new and efficient therapeutic strategies against HCC. Recently, traditional Chinese medicine has received increasing attention due to its broad-spectrum anti-inflammatory and anti-cancer activities. In particular, triptolide (TP), a biological diterpenoid trienpenoid isolated from the plant Tripterygium wilfordii, is a promising chemotherapeutic agent\(^11\). Studies suggest that low concentrations of TP can effectively inhibit the growth of malignant cancers including HCC\(^12\)\(^13\)\(^14\). Nonetheless, its clinical application has been limited by its severe systemic toxicities, low solubility, narrow therapeutic window, and unclear therapeutic targets\(^15\)\(^16\)\(^17\). Therefore, there is an urgent need to develop a TP delivery system for treatment of HCC with high efficiency and low toxicity.

Over the past decade, stimuli-responsive nanomedicine has experienced intensive development in pursuit of better therapeutic potency and attenuated toxicity\(^18\)\(^19\)\(^20\)\(^21\). The advantages of stimuli-responsive nanomedicine include excellent stability, improved tumor accumulation, desirable endocytosis, as well as preferable drug release capacity\(^22\)\(^23\)\(^24\)\(^25\). Particularly, these systems are capable of releasing therapeutic cargos responsive to not only endogenous stimuli (e.g., pH and ROS, etc. in the microenvironment of tumors), but also external stimuli (temperature, electromagnetic radiation and acoustic ultrasound, etc.)\(^26\)\(^27\). Light-responsive liposomes are especially attractive due to their controllability, availability and low toxicity\(^28\)\(^29\). For example, while photosensitizes were almost non-toxic to the human body, they generated ROS within the liposomes under light irradiation\(^30\)\(^31\). ROS plays a direct role in the process of photodynamic therapy (PDT)\(^31\)\(^32\), where it causes peroxidation of unsaturated phospholipids and, thus, renders them hydrophilic, resulting in the release of encapsulated drugs\(^26\)\(^33\). Ce6, a near-infrared (NIR) photosensitizer, can produce ROS under 650 nm laser irradiation for PDT. Therefore, the combined application of Ce6 and TP will benefit from the reduced toxicity via light controlled rapid release of TP specifically at the tumor site. Moreover, a patient-derived tumor xenograft (PDX) model of HCC (PDXHCC) was used to explore the potential of TP/Ce6-LP under light irradiation for effective anti-tumor therapy. The PDX model had the following advantages that could not only maintain biological stability and intratumoral heterogeneity, but also regenerated a microenvironment similar to the human tumor (for example, tumor vasculature, extracellular matrix and macrophages)\(^34\)\(^35\). Therefore, the PDX model could more accurately evaluate the therapeutic effect of nanomedicine.

Herein, we designed photo-activatable liposomes incorporating Ce6 and TP (TP/Ce6-LP) for HCC therapy (Scheme 1). First, the structure of the allylic hydrogens in egg yolk lecithin (PC-98T) could react with ROS generated by Ce6 to form reversible lipid peroxides upon near-infrared (NIR) laser irradiation, making the lipoasomal components hydrophilic to release TP. Afterwards, the oxygen-dependent PDT and TP-based chemotherapeutics exerted potent anti-tumor effect on the PDXHCC model. In addition, free TP caused severe liver damage, while TP/Ce6-LP reduced the hepatotoxicity of it. Therefore, our proposed TP/Ce6-LP was envisioned to not only enhance therapeutic efficiency but also reduce toxic effects of TP. Finally, TP/Ce6-LP under light irradiation possibly induced cell apoptosis via a caspase-3/PARP signaling pathway.

2. Materials and methods

2.1. Material

Triptolide was purchased from Chengdu Biopurify Phytochemicals Ltd. Cholesterol (Chol), distearoyl phosphatidylglycerole (DSPG) and egg yolk lecithin (PC-98T) were sourced from AVT (Shanghai) Pharmaceutical Tech Co., Ltd., Shanghai, China. GSH and GSSG assay kit, Annexin V-FITC/Propidium iodide (PI) Apoptosis Detection Kit, Cell Cycle and Apoptosis Analysis Kit, Bicinchoninic Acid (BCA) Protein Assay Kit and goat anti-rabbit IgG/HRP antibody were obtained from Beyotime Institute of Biotechnology, Nanjing, China. Reactive Oxygen Species Assay Kit was purchased from Beijing Solarbio Science & Technology Co., Ltd., Beijing, China. RPMI 1640 medium, trypsin, and fetal bovine serum (FBS) were provided by Gibco BRL, USA. Anti-caspase-3 antibody and anti-PARP-1 antibody were sourced from Cell Signaling Technology, USA. Anti-GAPDH (glyceraldehyde-
3-phosphate dehydrogenase) rabbit monoclonal antibody was purchased from WuXi AppTec, Shanghai, China.

2.2. Preparation of liposome

The ratio of Chol:DSPG:PC-98T was 1:1:1 in the blank liposomes. LPs loaded with TP and Ce6 (TP/Ce6-LPs) were prepared as the following: first, 30 mg of lipid materials, 4 mg TP, and 0.06 mg Ce6 were dissolved in 12 mL of a mixed organic solvent (chloroform:methanol = 5:1, v/v); then, the solvent was removed using a spin evaporator under reduced pressure at 60°C; next, the dry mixture was hydrated with 5 mL water under sonication and then the mixture was extruded at room temperature; finally, the obtained TP/Ce6-LPs were dialyzed (MWCO = 3500 Da) for 6 h to remove the free drug.

2.3. Characterization

The morphology and size of LPs were measured by using an EOL JEM-1011 transmission electron microscope (TEM). The size distribution and zeta potential of LPs were determined by dynamic light scattering analysis (DLS, Malvern Nano ZS90, UK). The absorbance and fluorescence spectra of liposomes were obtained using an ultraviolet—visible spectrometer (UV—Vis, UV-2450PC, Shimadzu, Japan) and fluorescence Spectrometer (LS50B, PerkinElmer, USA), respectively.

To determine the lipid peroxidation, the formation of conjugated dienes was assessed by spectrophotometry. The phototriggered lipid peroxidation reaction was measured in both ethanol, where the lipid and Ce6 were dispersed, and in PBS, where Ce6 was encapsulated in the lipid bilayer (TP/Ce6-LP). The solution was irradiated at 650 nm, 0.5 W/cm², and the absorption at 200–300 nm was monitored.

To determine the release profile of TP, the TP/Ce6-LP solutions were first treated under different conditions: 1) 650 nm NIR irradiation at a power density of 0.5 W cm⁻² for 5 min, 2) 100 μmol/L of H₂O₂, and 3) PBS solution. Then, the solution was transferred into a dialysis bag (MWCO = 3500 Da) placed in 100 mL related release medium at 37 °C under constant shaking. At indicated time points, 2 mL of release medium was removed for further measurement, followed by the addition of 2 mL release medium to keep the total volume constant. The amount of released TP was measured by using HPLC.

2.4. Singlet oxygen generation

Singlet oxygen generation of photo-triggered liposome in vitro was measured by DPBF(1,3-diphenylisobenzofuran). The TP/
Ce6-LP with or without VC mixture were mixed with 20 μmol/L DPBF, followed by 3 min irradiation with a power intensity (650 nm laser, 0.5 W/cm²). The absorbance of DPBF at 415 nm was monitored during the irradiation. Singlet oxygen generation of photo-triggered liposome in cells was measured by 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA). HepG2 cells were seeded in 6-well plates and cultured for 12 h, followed by treatment with TP/Ce6-LP at a Ce6 concentration of 5 μmol/L for 4 h. Then, the TP/Ce6-LP were divided into two parts, which were exposed under 3 min irradiation (650 nm laser, 0.5 W/cm²) or without irradiation.

2.5. **Cellular uptake**

HepG2 cells (5 × 10⁵) were seeded in 6-well plates in 1640 medium and cultured for 12 h. After treated with PBS for 8 h, Ce6 for 8 h, TP/Ce6-LP for 2, 4, and 8 h, respectively, the cells were fixed with 4% (w/v) paraformaldehyde for 30 min. Then, the cell nucleus was stained with DAPI (blue) for 5 min. Next, the actin cytoskeleton of cells was stained with FITC phalloidin (green) for 30 min. Last, the cells were visualized under confocal laser scanning microscopy (CLSM). For flow cytometry study, HepG2 cells (2 × 10⁵) were seeded in 6-well plates in 1640 medium and

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**Figure 1** Characterization of photosensitive liposomes. (A) TEM images of TP/Ce6-LP and coalescent TP/Ce6-LP after 3 min irradiation (650 nm, 0.5 W/cm²). Size distribution(B) and Zeta potential (C) of TP-LP, Ce6-LP and TP/Ce-LP, data are expressed as median, n = 3. (D) UV–Vis spectra of TP-LP, Ce6-LP, TP/Ce-LP and free Ce6. (E) Fluorescence spectra of Ce6 in DMSO, Ce6 in PBS and TP/Ce6-LP. UV–Vis spectra of TP/Ce6-LP (F) and PC-98T and Ce6 mixture (G) after 0, 0.5, 1, 2, 4, 6, 8, 10 min laser irradiation. (H) UV–Vis spectra of separate PC-98T solution and Ce6 solution after 0 and 10 min laser irradiation. (I) Release profile of TP from TP/Ce6-LP with or without periodic irradiation (650 nm, 0.5 W/cm²) and treatment with H2O2 (100 μmol/L). Data are presented as mean ± SD, n = 3. (J) ROS generation measurements by DPBF assay with or without laser irradiation (650 nm, 0.5 W/cm²). (K) ROS generation in HepG2 cells measured by CLSM. Scale bar = 20 μm.
cultured for 12 h. Then the cells were treated with TP/Ce6-LP (Ce6 concentration: 5 μmol/L) at 37°C for 2, 4 and 8 h, respectively. The collected cells were resuspended with PBS for detection by Flow Cytometry (FCM).

2.6. Cell viability studies

HepG2 cells (5 × 10^3) and 7404 cells (5 × 10^3) were seeded in 96-well plates in 1640 medium and cultured for 12 h. The medium was replaced with culture medium with the following drugs: TP/Ce6-LP, P/Ce6-LP-L, TP-LP+L, TP-LP-L, Ce6-LP+L, triptolide (TP), platinum (Pt), sorafenib (So), doxorubicin (Dox) at the concentration from 0.00625 to 50 μmol/L. After incubating for 24 h, the TP/Ce6-LP, TP-LP and Ce6-LP were irradiated with 650 nm light for 3 min. After 24 h incubation, 5 mg/mL MTT solution in PBS was added and the plates were incubated for another 4 h at 37°C. The cells were incubated another 12 h after irradiation and harvested to stain with annexin-V FITC and PI according to the manufacturer’s protocol for flow cytometry. Then the data were analyzed with FlowJo software.

2.8. Tissue distribution

BALB/c nude mice (18–20 g) were purchased from Guangdong Medical Laboratory Animal Center (Guangdong, China). All animal studies were carried out under Institutional Animal Care and Use Committee approved protocols of Southern Medical University. For in vivo fluorescence imaging, the subcutaneous patient-derived xenograft (PDX) model of HCC was established. When the tumor size reached of 150 mm³, mice were intravenously injected with free Ce6 and TP/Ce6-LP. The photos were taken at 4, 8 and 24 h after the drug injection. In vivo fluorescence imaging was performed on an IVIS Lumina III Imaging System (Caliper, USA) with excitation filter of 670 nm and emission filter of 700–800 nm. After the mice were sacrificed, tumor and tissues were excised and subjected to ex vivo fluorescence imaging and tissue section staining.
2.9. In vivo anticancer efficacy

PDX tumor-bearing mice established as described above were randomly divided into PBS, So, TP, Ce6-LP+L, TP/Ce6-LP+L and TP/Ce6-LP+L+L groups (n = 5). At Days 0, 2, 4, and 6, the mice were administered intravenously with drugs at the 10 mg/kg dose of So, 0.04 mg/kg dose of Ce6 and 0.4 mg/kg dose of TP. Ce6-LP+L and TP/Ce6-LP+L groups were irradiated with light at 650 nm (0.5 W/cm², 10 min) at Days 1, 3, 5 and 7. Tumor length (L) and width (W) were measured with calipers every day, and the tumor volume (V, mm³) was calculated. The body weight of each group was monitored every day as an indicator of systemic toxicity. The mice were sacrificed after therapeutic experiments and the tumors were weighted. The blood of mice was collected by eyeball blood collection method. Then tumors and major organs including heart, liver, spleen, lung, kidney were collected and kept in 4% (w/v) paraformaldehyde overnight for hematoxylin and eosin (H&E) staining, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay. In addition, the fresh tumors excised from mice were prepared for Western blotting.

2.10. Western blotting

Total proteins were extracted from tumor tissues. BCA protein assay kit was used to determine the protein concentration of each sample. Then equal amount protein samples were scraped into SDS-PAGE gel electrophoresis, followed by electro-transferring to PVDF membrane. These membranes were incubated with the primary antibodies at 4 °C overnight and horseradish peroxidase-conjugated secondary antibodies for 2 h. The immune complexes of each group were detected by ECL western blotting detection system.

2.11. Detection of blood biochemical indexes

The blood sample of mice was collected and centrifuged at 5000 rpm (Eppendorf Centrifuge 5424) for 5 min to obtain plasma samples for measuring the clinic parameters including heart indices containing creatine kinase (CK), creatine kinase-MB (CK-MB), and lactate dehydrogenase (LDH), liver-related alanine aminotransferase (ALT) and aspartate aminotransferase (AST).

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Figure 3 In vitro evaluation of anticancer activity. (A) Relative cell viability of HepG2 and 7404 cells after 48 h incubation of TP/Ce6-LP+L, TP/Ce6-LP-L, TP-LP+L, TP-LP-L, Ce6-LP+L, Triptolide (TP), Cisplatin (Pt), Sorafenib (So), Doxorubicin (Dox). (B) The Annexin-V/PI apoptosis assay of HepG2 cells after treated with each group for 24 h measured by FCM analysis.
and kidney-associated blood urea nitrogen (BUN) and creatinine (Cr) by an automatic biochemical analyzer.

2.12. Statistical analysis

The experimental data expressed as mean ± SD was analyzed using one-factor analysis of variance (SPSS software, version 22.0, SPSS Inc.). Statistical difference between various experimental and control groups was analyzed by Student’s t-test or ANOVA analysis. Differences were considered statistically significant at a level: *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$.

3. Results

3.1. Preparation and characterization of TP/Ce6-LP

The lipid film Ce6 and TP were hydrated with aqueous solution to prepare photo-sensitive liposome (LP). The drug encapsulation efficiency (DEE) of TP was 22.4% (TP-LP) and 26.7% (TP/Ce6-LP). The DEE of Ce6 was 91.7%. The drug-loading content (DLC) of TP was estimated to be 3.0% (TP-LP) and 3.6% (TP/Ce6-LP, w/w). The DLC of Ce6 was estimated to be 0.18%. The hydrodynamic diameters of TP-LP, Ce6-LP and TP/Ce6-LP were measured as 71.22, 72.88, and 109.97 nm by dynamic light scattering (DLS, Fig. 1B). Concurrently, the zeta potential of these three liposomes were determined as ~56.67, ~61.47 and ~56.33 mV (Fig. 1C). We examined the morphology of TP/Ce6-LP before and after the laser irradiation by transmission electron microscopy (TEM, Fig. 1A). According to the TEM, TP/Ce6-LP possessed the spherical structure. TP/Ce6-LP is oxidized and their structural integrity was destroyed after laser irradiation. The absorption and fluorescent spectra of TP/Ce6-LP and Ce6-LP indicated that Ce6 was successfully encapsulated in the liposomes, verifying that the near infrared emissions from TP/Ce6-LP could motivate the PDT process and trigger the release of TP (Fig. 1D and E). The peroxidation of unsaturated phospholipids experiment has been assessed by following the absorption spectra. Upon irradiation of Liposomes, the absorption peak at 238 nm increased with time, indicating the occurrence of lipid peroxidation (Fig. 1F). Irradiation of PC-98T and Ce6 mixture also has the above phenomenon (Fig. 1G). However, when PC-98T and Ce6 were irradiated, respectively, there were negligible changes at 238 nm (Fig. 1H). These results proved that peroxidation of unsaturated phospholipids was triggered by the NIR-induced ROS.

To demonstrate the light-responsive behavior of TP/Ce6-LP, the drug release of TP was conducted in dark or under NIR irradiation and with 100 μmol/L H2O2 (Fig. 1I). Results show that TP/Ce6-LP exhibited a sustained release and limited TP release in the absence of NIR light as well as in H2O2 (37% and 32% of TP released) at 1000 min. Under NIR irradiation, 98% of TP was rapidly released from nanoparticles in 1000 min. These results show that liposomes could be released under light irradiation. To confirm the ROS generation that could trigger the oxidation of unsaturated phospholipid under irradiation, the in vitro generation of singlet oxygen was evaluated by DPBF. We observed that ROS production increased rapidly over an irradiation time in 6 min (Fig. 1J). To further confirm the ROS generation can be possible in cells, in situ ROS production in HepG2 cells was measured by DCFH-DA using CLSM (Fig. 1K). Results demonstrate that HepG2 cells treated with TP/Ce6-LP follow by irradiation produced more ROS than other treatment group.
3.2. Cellular uptake of TP/Ce6-LP

As Ce6 is fluorescent, Ce6 fluorescence was applied to track the internalization of nanoparticles via CLSM and FCM in HepG2 cells. TP/Ce6-LP entered tumor cells through an endocytosis pathway, leading to the intercellular accumulation of drugs for promoting the drug uptake. The results showed a time-dependent increase in TP/Ce6-LP uptake on the HepG2 cells for different time points (Fig. 2A). In addition, TP/Ce6-LP achieved remarkable higher drug uptake than free Ce6. Further quantitative results by flow cytometry also showed that the fluorescence intensity in HepG2 cells increased 1.7-fold after 8 h treatment with TP/Ce6-LP than cells in PBS (Fig. 2B and C).

3.3. Proliferation inhibition

The cytotoxicity of TP/Ce6-LP in comparison to various agents was evaluated by MTT assay on HepG2 and 7404 cells under light irradiation or without light irradiation (Fig. 3A). Upon 650 nm light irradiation, the cell viability of TP/Ce6-LP decreased with the increase of TP concentration, exhibiting the most remarkable effect to kill cancer cells compared with free TP. This synergy also confirmed by cell apoptosis evaluation. Determination of the apoptosis on HepG2 cells incubated with the same experimental and control agents showed similar trends of MTT results (Fig. 3B). TP/Ce6-LP followed by NIR irradiation (75% cell apoptosis) significantly increased cell death compared with TP (36% cell
apoptosis), confirming that TP/Ce6-LP possessed excellent therapeutic effect.

### 3.4. Biodistribution study

Improved accumulation in tumor is critical for the ultimate therapeutic consequence. The biodistribution of TP/Ce6-LP and Ce6 in BALB/c nude mice bearing PDX model of hepatocellular carcinoma was determined by real-time IVIS imaging. The Ce6 fluorescence appeared to persist in tumors despite 24 h post administration in TP/Ce6-LP group, but in Ce6 group it has disappeared (Fig. 4A). Ex vivo imaging and semi-quantitative analysis for the major organs also suggested that TP/Ce6-LP had markedly stronger fluorescence compared to Ce6 (Fig. 4B). After 24 h administration, the isolated organs and tumor tissues were stained to observe the drug distribution. The results find that the fluorescence intensity of TP/Ce6-LP group was much higher than that of Ce6 group in the tumor site (Fig. 4C). These results show that TP/Ce6-LP possess appreciable tumor accumulation and retention for a wide treatment window.

### 3.5. Antitumor efficacy on PDX model of HCC

Free drug and drug-loading photo-activatable liposomes were tested on BALB/c nude mice bearing PDX model of hepatocellular carcinoma to explore the advantages of drug releasing platform for tumor. Herein, when the tumor volume reached about 50 mm³, the mice were treated with diverse therapeutic groups (Fig. 5A). Compared with PBS group, So, TP, Ce6-LP+L and TP/Ce6-LP-L groups have some anti-tumor effects. However, TP/Ce6-LP+L exhibited best antitumor effect, which significantly inhibited the tumor volume to 276 mm³ after 12 day (Fig. 5B and C). In addition, the tumor weight in TP/Ce6-LP+L group was only 4% of the PBS group, which showed the significant tumor suppression (Fig. 5D). During the treatment period, all groups showed no obvious weight loss, indicating that our photo-activatable liposomes had good biocompatibility (Fig. 5E). These data demonstrate that TP delivered by the adjustable photo-activatable liposome dominated the synergistic therapy and show great therapeutic advantages in HCC tumor. In addition, TP/Ce6-LP+L group displayed much better antitumor effect than PBS group according to the significant histological damages in the tumor sections validated by the hematoxylin and eosin (H&E, Fig. 5F), consisting with the above in vivo antitumor effects.

### 3.6. Molecular mechanism of photo-activatable synergistic therapy

The potent anti-tumor efficacy of TP/Ce6-LP was further validated by TdT-mediated dUTP Nick-End Labeling (TUNEL)-staining of tumor tissue sections (Fig. 6A). It was found that the degree of apoptosis in PBS group was lower, while that in TP/Ce6-LP+L group was the highest and the relative level appeared in the following order: PBS<So< TP<Ce6-LP+L<TP/Ce6-LP-L<TP/Ce6-LP+L. Further Western blotting quantification analysis after treatment with each group were also performed (Fig. 6B and C). These results show that TP/Ce6-LP+L produced more...
CLEAVED CASPASE-3 in tumor tissue, and the relative level appeared in the following order: PBS < So < TP < Ce6-LP + L < TP/Ce6-LP-L < TP/Ce6-LP. CLEAVED-PARP1 expression was in the same order, suggesting that TP/Ce6-LP induced cell apoptosis through increasing CLEAVED CASPASE-3 and CLEAVED-PARP1 expression.

3.7. Application safety evaluation

The blood biochemistry analysis (Fig. 7A–G) on the BALB/c mice and H&E examination of the normal organs (Fig. 7H) collected from mice receiving different treatments for 12 d were determined for evaluation of application safety. There were no
obvious changes in major organs after treatment with drug-loading liposomes, verifying an excellent biocompatibility. However, the blood liver function indexes (ALT and AST) of TP group were higher than that of PBS group, indicating that TP induced liver function injury. Moreover, H&E staining pictures of liver in TP group showed scattered apoptotic bodies, round apoptotic cells, deep nuclear staining and cytoplasmic condensation, indicating that TP was harmful to the liver. These results demonstrated that drug-loading liposomes reduced side effects of TP.

4. Discussion

HCC has unfavorable prognosis for survival as it is poorly responsive to current therapy. At present, the main chemotherapeutic agent clinically used in the treatment of HCC is sorafenib. Sorafenib is included in the clinical treatment of HCC as the first-line agent in 2008, owing to effective suppression effect of both tumor cell proliferation and tumor angiogenesis. However, with nonspecific distribution in vivo and insufficient dosage in the tumor site, sorafenib can only achieve average survival by two months, which results in its highly restricted clinically. TP has been considered to be a more promising therapeutic agent against HCC than sorafenib. However, its high toxicity and poor solubility limit its clinical application. Thus, reducing the side effects and improving therapeutic index of TP should be the direction of our efforts in the treatment of HCC.

Nanomedicine derived from nano-size drug delivery systems has significantly improved drug delivery to specific cells or tissues, including stimuli-responsive nanoparticles. Growing evidence shows that stimuli-responsive nanoparticles have considered as a drug delivery system for targeted intracellular controlled release delivery of chemotherapeutic agents. Currently, it has attracted more attention for clinical therapy. Therefore, we synthesized a photo-activatable liposomes integrated Ce6 and TP (TP/Ce6-LP) for HCC targeted therapy. The TP/Ce6-LP+L systems were characterized by excellent stability, improved tumor accumulation, desirable endocytosis, as well as preferable drug release capacity, which also verified by drug particle size stability test, drug release test, cell endocytosis test.

In our study, we have observed that TP/Ce6-LP+L has stronger anti-HCC efficacy than free TP in vivo. This result is probably due to several advantages of TP/Ce6-LP+L. Firstly, as compared to normal tissues, TP/Ce6-LP+L improved tumor accumulation through the EPR effect, resulting in a stronger anti-HCC efficacy. Secondly, with NIR irradiation, ROS is released by photosensitizers (Ce6) within TP/Ce6-LP. Thirdly, the released ROS plays a direct role in the process of photodynamic therapy (PDT). Fourthly, the allylic hydrogen structure in unsaturated phospholipid reversibly formed hydrophilic lipid peroxides upon intermittent near-infrared light-induced ROS, thus facilitating on-demand release of TP prodrugs from liposomes to enter the tumor cells. Last but not the least, the toxicity of TP was notably reduced following its encapsulation in TP/Ce6-LP. Taken together, TP/Ce6-LP+L achieved ON/OFF drug release, thereby reducing tumor sizes in PDX model, suggesting its targeting and control potential. Furthermore, in order to clarify its molecular mechanism, the whole lysate was extracted from tumor tissues for Western blot assay. More interestingly, cleaved-caspase-3 and cleaved-PARP1 were markedly up-regulated in PBS group, while tumor treated with TP/Ce6-LP+L had the highest protein levels. These results demonstrate that TP/Ce6-LP+L mediated apoptosis through caspase-3/PARP signaling.

5. Conclusions

In summary, a preferable photo-activatable drug release capacity of both tumor cell proliferation and tumor angiogenesis. However, with nonspecific distribution in vivo and insufficient dosage in the tumor site, sorafenib can only achieve average survival by two months, which results in its highly restricted clinically. TP has been considered to be a more promising therapeutic agent against HCC than sorafenib. However, its high toxicity and poor solubility limit its clinical application. Thus, reducing the side effects and improving therapeutic index of TP should be the direction of our efforts in the treatment of HCC.

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In our study, we have observed that TP/Ce6-LP+L has stronger anti-HCC efficacy than free TP in vivo. This result is probably due to several advantages of TP/Ce6-LP+L. Firstly, as compared to normal tissues, TP/Ce6-LP+L improved tumor accumulation through the EPR effect, resulting in a stronger anti-HCC efficacy. Secondly, with NIR irradiation, ROS is released by photosensitizers (Ce6) within TP/Ce6-LP. Thirdly, the released ROS plays a direct role in the process of photodynamic therapy (PDT). Fourthly, the allylic hydrogen structure in unsaturated phospholipid reversibly formed hydrophilic lipid peroxides upon intermittent near-infrared light-induced ROS, thus facilitating on-demand release of TP prodrugs from liposomes to enter the tumor cells. Last but not the least, the toxicity of TP was notably reduced following its encapsulation in TP/Ce6-LP. Taken together, TP/Ce6-LP+L achieved ON/OFF drug release, thereby reducing tumor sizes in PDX model, suggesting its targeting and control potential. Furthermore, in order to clarify its molecular mechanism, the whole lysate was extracted from tumor tissues for Western blot assay. More interestingly, cleaved-caspase-3 and cleaved-PARP1 were markedly up-regulated in PBS group, while tumor treated with TP/Ce6-LP+L had the highest protein levels. These results demonstrate that TP/Ce6-LP+L mediated apoptosis through caspase-3/PARP signaling.

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Author contributions

Zhiquiang Yu and Shijian Zhang designed the research. Ling Yu carried out the experiments and performed data analysis. Zhenjie Wang, Zhuomao Mo and Binhua Zou participated part of the experiments. Yuanyuan Yang, Rui Sun and Wen Ma provided experimental drugs and quality control. Ling Yu and Zhenjie Wang wrote the manuscript. Meng Yu and Zhiquiang Yu revised the manuscript. All of the authors have read and approved the final manuscript.

Conflicts of interest

The authors have no conflicts of interest to declare.

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