Activation of CYP3A by ABC phenomenon potentiates the hepatocellular carcinoma-targeting therapeutic effects of PEGylated anticancer prodrug liposomes

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ABC phenomenon potentiates anti-HCC efficacy

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Abbreviations

ABC, accelerated blood clearance; AUC \((0\rightarrow30\,\text{min})\), area under the plasma concentration-time curve from time 0 to 30 min time point; AUC \((0\rightarrow10\,\text{h})\), area under the plasma concentration-time curve from time 0 to 10 h time point; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; AFP, alpha-fetoprotein; CP, Cyclophosphamide; CYPs, cytochrome P450s; DSPE-PEG\(_{2000}\), Distearoyl phosphatidylethanolamine-polyethylene glycol 2000; DiR, 1,1-dioctadecyl-3,3,3,3-tetramethylindotricarbocyanine iodide; DEN, diethylnitrosaminem; DEX, Dexamethasone; EPR, enhanced permeability and retention; GGT, serum γ-glutamyl transpeptidase; HA, hepatic accumulation; IS, internal standard; KCZ, Ketoconazole; MRM, multiple reaction monitoring; MRT\(_{(0\rightarrow10\,\text{h})}\), mean residence time from time 0 to 10 h time point; NPs, Nanoparticles; PEG-L, PEGylated liposomes; PCS, photon correlation spectroscopy; PBS, phosphate-buffered saline; PEG, polyethylene glycol; PEG-CP-L, PEGylated liposomal cyclophosphamide; PEG-B-L, blank PEGylated liposomes; SA, splenic accumulation; PEG-DiR-L, PEGylated liposomal DiR; PDI, polydispersity index; SD, standard deviation; SD rats, Sprague-Dawley rats; TEM, transmission electron microscopy; t\(_{1/2}\), half-life; Vz, apparent volume of distribution.
Abstract: Reduced enzyme activity in hepatocellular carcinoma (HCC) and poor targeting limit the application of enzyme-activating prodrugs, which is also detrimental to the effective treatment of HCC. Here, we investigated whether accelerated blood clearance (ABC) phenomenon occurs in HCC models following repeated injections of PEGylated liposomes (PEG-L), thus inducing prodrugs accumulation and activation in the liver and exerting highly effective and low-toxicity therapeutic effects on HCC. Firstly, PEGylated liposomal cyclophosphamide (PEG-CP-L) was prepared by solvent injection and characterized. Importantly, pre-injection of PEG-L induced ABC phenomenon and activation of CYP3A in both HCC rats and HCC mice by investigating the effects of repeated injections of PEG-L on pharmacokinetics and tissue distribution. Next, the efficacy and toxicity of repeated injections of PEG-L in HCC mice were examined, and our data indicate that repeated injections are administered in a manner that significantly enhances the antitumor effect compared to controls with little or no toxicity to other organs. To further reveal the pharmacokinetic mechanism of PEG-L repeated administration for the treatment of HCC, the protein expression of hepatic CYP3A and the concentration of CP in the liver and spleen of HCC mice by inhibiting CYP3A were analyzed. These results revealed that inducing CYP3A to accelerate the rapid conversion of prodrugs that accumulate significantly in the liver is a key mechanism for the treatment of HCC with repeated injections of PEG-L. Collectively, this work taps into the application potential of the ABC phenomenon and provides new insights into the clinical application of PEGylated nanoformulations.

Keywords: Hepatocellular carcinoma; anticancer prodrugs; PEGylated liposomes; Accelerated blood clearance phenomenon
Significance statement: This study revealed that repeated injections of PEG-L could induce the ABC phenomenon characterized by hepatic accumulation and CYP3A activation based on HCC rats and HCC mice. Furthermore, it was verified that induction of the ABC phenomenon dependent on hepatic accumulation and CYP3A activation could enhance the anti-hepatocellular carcinoma effects of PEGylated anticancer prodrugs in HCC mice. This elucidated the relevant pharmacokinetic mechanisms and unearths new clues for solving the clinical application of PEGylated nanoparticles.

1. Introduction

Primary liver cancer, arising from genetic and epigenetic alterations, is the sixth most common cancer and the second leading cause of cancer-related mortality worldwide (Foerster et al., 2022; Llovet et al., 2022; Ladd et al., 2023). Particularly, hepatocellular carcinoma (HCC) is responsible for about 80% of primary liver cancers. While over two-thirds HCC patients are diagnosed at advanced stages that do not obtain long-term benefit from surgical resection, embolization and ablation (Bao and Wong, 2021; Yao et al., 2021). Sorafenib and Lenvatinib are the first line of chemotherapy for advanced HCC, but sufferers who taking them have reported a median overall survival of ~11-14 months (Llovet et al., 2022). The reason why HCC is difficult to cure is that in addition to the low lethality, there is no way to efficiency deliver anti-cancer drugs to the liver lesions (Giraud et al., 2021). Furthermore, a number of anti-cancer prodrugs have strong cytotoxicity after activation, but fail to effectively recognize tumor cells and are prone to acquired resistance (Bildstein et al., 2011; Ladd et al., 2023). Thus, identifying the best therapeutic options and develop effective strategies still represents a major challenge.

Polyethylene glycol (PEG) is a polyether consisting of ethoxy units derived from the
ring-opening polymerization of ethylene oxide. Nanoparticles (NPs) conjugated with linear PEG via functional groups possess stealth characteristics that avoid recognition and uptake by the reticuloendothelial system (RES) (Shi et al., 2021; Ibrahim et al., 2022). Nevertheless, it was found that repeated injections of PEGylated liposomes (PEG-L) fail to have long-circulation properties in the blood and were accompanied by increased hepatic aggregation, a phenomenon known as accelerated blood clearance (ABC) (Shiraishi et al., 2016; Su et al., 2018). Efforts have been made in recent years to eliminate the adverse outcomes associated with the ABC phenomenon, including altering the injection protocol, lipid composition, size, and surface charge of liposomes (Laverman et al., 2001; Suzuki et al., 2014; Zhang et al., 2017; Zhang et al., 2021). Thinking differently, in light of the hepatic accumulation properties of the ABC phenomenon, researchers injected PEGylated organic-inorganic hybrid nanoparticles into the mouse model of hind limb ischemia twice, effectively avoiding the accumulation of drugs in nanomaterials in ischemic tissues of peripheral arterial diseases (Im et al., 2016), indicating that the clinical application of PEG-L has great potential value and deserves to be explored in depth.

CYP450 enzymes exert biotransformation of conventional chemotherapeutic drugs and can increase the pharmacological activity of cytostatic precursor drugs (Walther et al., 2017; Wang et al., 2023). CYP3A, a major subfamily of the cytochrome P450 superfamily, have been reported to be involved in the metabolism of 45-60% of all drugs on the market (Klyushova et al., 2022). Studies have shown that CYP3A expression is significantly lower in HCC tissues than in para-cancerous tissues, which directly affects the exposure of enzyme substrates, including anticancer prodrugs, in tumor tissues (Flannery et al., 2020; Klyushova et al., 2022; Wang et al., 2023). Previously, we published a study in DMD reporting that a combination of pharmacokinetic,
biodistribution, mRNA and protein analyses of CYP in rats revealed for the first time that repeated injections of PEG-L induced an increase in the activity and expression of CYP3A enzymes (Wang et al., 2019a; Wang et al., 2019b). Thus, if the drug contained in PEG-L is a metabolic substrate for CYP3A, then CYP3A activated by the ABC phenomenon accelerates the metabolism of the drug, thereby altering the tissue distribution and metabolic behavior of the re-entering PEG-L.

Cyclophosphamide (CP) is both an anticancer and immunosuppressive prodrug that activated by CYP3A1 enzyme to form its pharmacologically active metabolite 4-hydroxycyclophosphamide (Hall et al., 2018; Shanker Kasudhan et al., 2022). Unfortunately, some deficiencies restricted its clinical application, including low targeting and toxicity (Dionísio et al., 2022; Mombeini et al., 2022). Therefore, this study attempts to encapsulate CP in PEG-L, which could accelerate the conversion of CP and increase the concentration of effective drugs in tumor tissues by inducing the ABC phenomenon to achieve low-toxicity and highly effective targeted therapy for HCC. Firstly, it was examined whether animals under the pathological model of HCC cause ABC phenomenon characterized by liver aggregation and CYP3A activation. Then, the anti-hepatocarcinogenic effect of PEGylated cyclophosphamide liposomes (PEG-CP-L) was investigated by repeated injections in HCC model mice. Finally, the pharmacokinetic mechanism of repeated injection of PEGylated precancerous liposomes for the treatment of HCC was revealed with the aid of CYP3A enzyme inhibitors to study the advantages from the perspective of hepatic accumulation of CP and CYP3A enzyme activation.

2. Materials and methods

2.1 Materials
Cyclophosphamide (CP, purity > 98%) and Antipyrine [internal standard (IS), purity > 98%] were provided by Shanghai Aladdin Biochemical Technology Co. (Shanghai, China). 1,1-dioctadecyl-3,3,3,3-tetramethylindotricarbocyanine iodide (DiR) was obtained from Dalian Meilun Biotechnology Co. (Dalian, China). Cholesterol was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Distearoyl phosphatidylethanolamine-polyethylene glycol 2000 (DSPE-PEG<sub>2000</sub>) was obtained from Shanghai Peng Shuo Biotechnology Co. (Shanghai, China). To determine CYP3A enzyme activity, Testosterone and 6β-hydroxytestosterone were purchased from Sigma Aldrich Co. (Missouri, USA). NADPH tetrasodium salt were obtained from MedChemexpress Biotechnology, Inc. (New Jersey, US). Ketoconazole (KCZ, purity >99.0%) and Dexamethasone (DEX, purity >99.0%) were provided by Shanghai Yuanye Biotechnology Co., Ltd. (Shanghai, China). For detecting mRNA, SYBR Green PCR Kit (Toyobo life sciences Co., Ltd., Shanghai, China), the forward and reverse primers of CYP3A and β-actin (Sangonn Biotech Co., Ltd., Shanghai, China), TRIzol reagent (Life Technologies Inc., Carlsbad), and Kit of RevertAid First Strand cDNA Synthesis (Thermo Fisher Scientific, Vilnius, Lithuania) were used. For measuring protein, RIPA lysis buffer (Shandong Scitech Biotechnology Co., Ltd., Shandong, China), BCA protein assay kit, HRP-conjugated goat anti-rabbit or mouse IgG and enhanced chemiluminescence reagent kit (Biosharp life sciences Co., Ltd., Hefei, China), mouse or rabbit antibodies of anti-CYP3A1, anti-CYP3A11 and anti-GAPDH (Abcam, Cambridge, UK) were used. LC-MS grade formic acid was obtained from Fluka Analytical (St. Louis, Missouri, USA). Deionised water was provided by Milli-Q Reference Ultrapure Water System (Merck, Darmstadt, Germany). Anhydrous ethanol (analytical purity) was obtained from Shanghai Runjie Chemical Reagent Co. (Shanghai, China). Methanol and acetonitrile were chromatographic purity.
In order to culture the cells, 1640 medium (Shandong Scitech Biotechnology Co., Ltd., Shandong, China) and fetal bovine serum (FBS; HyClone, California, USA) were used.

2.2 Cell lines and cell culture

Mouse H22 liver cancer cell line was purchased from the Guangzhou Jennio Biotechnology, where mycoplasma contamination detection and short tandem repeat profiling were performed for quality and identity guarantee. Cells were tested negative for mycoplasma contamination. Cells were maintained in a 37°C incubator with 5% CO₂, in 1640 medium, supplemented with 10% fetal bovine serum and 100 units/mL penicillin.

2.3 Establishment of HCC models

All animals lived in an environment maintained at room temperature conditions (25 ± 2°C) with a 12-hour light-dark cycle for 1 week before experiments, allowed free access to water and standard rodent diet. All animal experimental procedures were carried out in strict compliance with the principles of animal care as assessed and approved by the Institutional Animal Care and Use Committee of Anhui University of Chinese Medicine. This study was conducted in accordance with the National Research Council Guide for Care and Use of Laboratory Animals.

Male Sprague-Dawley (SD) rats (five to six weeks old, 220 ± 15 g) were induced by 0.5% aqueous diethylnitrosaminem (DEN) solution to establish a primary HCC model (Wu et al., 2016; Liu et al., 2020). The dose was set at 50 mg/kg and gavaged twice a week for the first four weeks, and then changed to once a week for 14 weeks. Besides, mouse HCC cells H22 (density of 1.0 ×10⁶ /mL) in logarithmic growth phase were injected subcutaneously into the axilla of male BALB/C mice (five to six weeks old, 20 ± 2 g). After the tumor mass grew, a tumor tissue of
approximately 1 mm$^3$ in size was taken to expose the liver of the recipient mouse and inoculated into the left outer lobe. The cotton was gently pressed for a few seconds, and the liver was returned to its original position and surgically sutured.

2.4 Preparation and characterization of PEG-L

2.4.1 Preparation of PEG-L

PEG-CP-L was prepared by the ethanol injection method. The preparation method was as follows. The required materials were weighed precisely according to the prescribed amount, and the egg yolk lecithin, cholesterol and PEG2000-DSPE were fully dissolved in a certain amount of anhydrous ethanol, and phosphate buffer solution (PBS, pH=7.4) was used as the aqueous phase. Set the speed of magnetic stirrer to 1000 rpm and the temperature to 50°C. When the organic and aqueous phases were heated to that temperature, the organic phase was injected into the aqueous phase uniformly and rapidly with a 1 mL syringe, and the reaction was continued under the same conditions for 2 h to obtain PEG-CP-L. After being cooled and stored in a refrigerator at 4°C, the whole preparation process was carried out under a light-proof environment. Blank PEGylated liposomes (PEG-B-L) were prepared using the same method as for PEG-CP-L except that CP was absent. For biodistribution studies, fluorescently labeled PEG-L was prepared by adding the hydrophobic fluorescent dye DiR (1 mol% liposomal phospholipid) to the lipid mixture.

2.4.2 Characterization of PEG-L

The mean particle size, Zeta potential and polydispersity coefficient (PDI) of the liposomes were determined by photon correlation spectroscopy (PCS) at 25°C, using a Zetasizer particle size analyser (Nano ZS90, Malvern Instruments, Malvern, UK). Transmission electron microscopy
(TEM, HT7700, JEOL Ltd., Japan) was used to observe the morphological appearance. A validated HPLC (Thermo Scientific™ UltiMate™ 3000, USA) method was used for the determination of drug content to calculate the encapsulation rate (EE%) of the liposomes. CP was separated by cosmosil C_{18}-ODS (4.6×250 mm, 5 µm) reverse column, the column temperature was set at 30°C, methanol: 0.1% formic acid aqueous solution (30: 70, v/v) was used as the mobile phase, and the flow rate was 1.0 mL/min. The ultraviolet detection wavelength was 254 nm. Each injection volume was 20 µL. The retention time of CP was around 6.20 min with no significant spurious interference near the peak. The calibration curve was linear at a range of 10.0-100.0 µg/mL with the corresponding linear regression equation of $Y = 4743X-3044$ and a correlation coefficient of $R^2 = 0.9995$.

2.5 Pharmacokinetics and tissue distribution after repeated injections of PEG-L

2.5.1 Determination of the pharmacokinetic curves of DiR

In order to figure out the pharmacokinetics of PEG-DiR-L in the plasma of HCC rats, HCC rats were randomly divided into single-injection and repeated injection groups (n = 6 for each group). The repeated injection group was pre-injected with PEG-B-L (0.05 µmol of PEG2000-DSPE/kg) 3 days before the last day of administration as our previous study (Wang et al., 2019a; Wang et al., 2019b), while the single injection group was given PBS for the first dose as a control, and rat plasma was taken at different time points after giving 1 mg/kg PEG-DiR-L. Plasma proteins were precipitated by methanol and the supernatant was centrifuged at high speed into a black 96-well plate to determine the fluorescence value of DiR (Ex=748 nm, Em=780 nm) in a multifunctional enzyme marker, indicating good linearity. The standard curve for the DiR in
vivo analytical method was \( Y = 95960.97X + 2.09 \) \( (R^2 = 0.9914) \) over the concentration range of 25-1000 ng/mL. The pharmacokinetic curves of DiR were plotted, and the main pharmacokinetic parameters such as area under the drug-time curve (AUC), mean retention time (MRT), total clearance (CLz), plasma half-life \( (t_{1/2}) \) and apparent volume of distribution (Vz) were calculated using DAS2.0.

2.5.2 Fluorescence imaging to determine the tissue distribution of DiR

To observe the distribution of PEG-DiR-L, HCC rats and HCC mice were divided into single injection and repeated injection groups \( (n=3 \) for each group). The repeated injection group was pre-injected with PEG-B-L \( (\text{containing } 0.05 \mu\text{mol of PEG2000-DSPE/kg}) \) 3 days before the last day of injection with 1 mg/kg of PEG-DiR-L, while the single injection group was given PBS for the first dose as a control. Based on the results of plasma pharmacokinetics, we chose to remove all organs at 1, 2 and 6 h after the last administration of HCC rats and place them on IVIS spectroscopy \( (\text{PerkinElmer, USA}) \) for fluorescence imaging. While HCC mice were detected of drug administration and migration at different time points after 1, 2, 6 and 12 h after the last administration of PEG2000-DSPE/kg. The fluorescence signal intensity of DiR was quantified by fluorescence emission \( (\text{Ex}= 748 \text{ nm, Em}= 780 \text{ nm}) \) using Living Image version 4.5.2 small animal imaging software.

2.6 Determination of CYP3A enzyme activity by in vitro incubation with liver microsomes

2.6.1 Establishment of analytical methods for 6β-hydroxytestosterone

An analytical method for 6β-hydroxytestosterone \( (\text{a metabolite of testosterone}) \) and internal standard \( (\text{IS, hydrocortisone}) \) was established by HPLC. The chromatographic separation system
was a cosmosil C18-ODS (4.6×250 mm, 5 µm) reverse column maintained at 25 °C. Isocratic elution was carried out with a liquid chromatography mobile phase consisting of 10 mmol/L Na₂HPO₄ in water and chromatographic grade methanol (48: 52; v/v) at a flow rate of 1.0 mL/min and an injection volume of 10 µL. The detection wavelength was 254 nm and the complete analysis time for one sample was 60 min. The linear regression equation for 6β-hydroxytestosterone over the concentration range of 1.25-20 μg/mL was Y = 0.0351X-0.0133 (R² = 0.9992).

2.6.2 Extraction of liver microsomes

Excess blood was removed from the liver by in situ single-channel hepatic perfusion, and animals in each group were anesthetized with 2.5% isoflurane. The hepatic portal vein was exposed for insertion of a 20G indwelling needle, the inferior vena cava was isolated, and perfusate (saline) was added to the perfusion system until the rat livers were flushed to an earthy yellow color by perfusion. The liver was clipped, and 1 g of liver was added to 9 mL of 0.1 mol/L PBS homogenate. The supernatant was obtained by differential centrifugation at 9000 ×g for 10 min, followed by centrifugation at 100000 ×g for 60 min to retain the micronized precipitate, and the supernatant was discarded and resuspended in low-temperature 30% glycerol PBS to obtain liver microsomes. The protein concentration of the extracted liver microsomes was quantified by the BCA method, and the protein concentration of the liver microsomes was adjusted to 12.5 mg/mL, which was placed in a -80°C refrigerator for backup.

2.6.3 Determination of CYP3A enzyme activity

The incubation system was divided into two groups, control and experimental, and the
experimental group was repeated three times. In the experimental group, each tube contained 10 μL 12.5 mg/mL normal liver microsomes, 5 μL 20 mmol/L testosterone solution, and the reaction system was made up to 480 μL with PBS. To start the reaction, 20 μL 10 mmol/L NAPDH solution was added after pre-warming in a 37 ℃ water bath for 2 min. The volume of the whole reaction system was 500 μL. After 20 min, the reaction was terminated by adding 3.5 mL of ice-cold ethyl acetate solution, and then 10 μL of IS solution (hydrocortisone, 500 µg/mL) was added. The control group did not add NAPDH to start the reaction, the rest of the procedure was the same as that of the experimental group. Before centrifugation at 3500 rpm for 10 min, the mixture was vortexed for 2 min to mix well, left at room temperature for 10 min. The top layer of ethyl acetate was evaporated in a vacuum drying oven, 200 μL of 50% methanol solution was re-dissolved, vortexed for 2 min and then centrifuged at 13000 rpm for 10 min, and the supernatant was taken into the sample and analyzed under the same conditions as “2.6.1”. The peak areas of 6β-hydroxytestosterone and IS were recorded to calculate the testosterone 6β-hydroxylase rate.

2.7 Western blot analysis for CYP3A

Tissue samples added to the RIPA lysis solution containing 1% protease inhibitor were well ground in a low temperature grinder and then lysed at 4°C for 1 h and then centrifuged at 14000 g for 10 min. Protein extracts were quantified by BCA and boiled by adding to the loading buffer to obtain protein samples. Then, protein samples were electrophoresed in a pre-made gradient gel and transferred to a nitrocellulose membrane. The membrane was closed with fast closing solution for 10 min at room temperature, washed with TBST and incubated in primary antibody at 4°C overnight. Next day, the membrane was washed and incubated with HRP-conjugated secondary
antibody at room temperature for 1 h. The membrane was developed using chemiluminescent solution. Western blot data in figures are all representative of more than three independent experiments.

2.8 RNA extraction and RT-qPCR for CYP3A

The extraction of total RNA from liver tissue was performed with TRIzol extraction reagent, and the quantification of RNA was performed by diluting the RNA 50 times with RNase-free water. Subsequently, RNA concentration (ng/mL) was assayed using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA) and the purity of RNA was determined based on the OD260/OD280 (R) values. The reverse transcription process was performed using the RevertAid First Strand cDNA Synthesis Kit according to the instructions of the Reverse Transcription Kit and using β-actin as an internal reference gene. The target gene was quantified by fluorescence using the SYBR Green PCR kit. The total reaction system was 20 μL for each sample, and the reaction program was set as follows: pre-denaturation at 95℃ for 10 min, denaturation at 95℃ for 15 s, annealing at 60℃ for 1 min (40 cycles), and the final melting curve was 95℃ for 15 s and 60℃ for 1 min. 2^-ΔΔCT method was used to analyze the relative gene expression differences.

2.9 ELISA method for the determination of Serum biochemical indicators

Alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), serum γ-glutamyl transpeptidase (GGT) and hepatocellular carcinoma-specific indicator alpha-fetoprotein (AFP) were measured by ELISA. The steps of the instructions were strictly followed. The diluted plasma samples were incubated with the detection antibodies at 37℃ for a
period of time and then the plates were washed with washing working solution. Next, Streptavidin-HRP working solution was added to each well and incubated at 37°C for a period of time. Then the reaction was terminated by adding TMB for color development for a few minutes and finally the OD value was derived in the Enzyme Markers, and the concentration of the different indicators was calculated according to the linear regression equation.

2.10 Immunohistochemical determination of Ki67

Paraffin-embedded representative tissue blocks containing tumors were cut into 4-μm-thick serial sections. The slices were dried in a desiccator and then treated transparently with xylene, followed by hydration with ethanol of different concentration gradients. After antigen high-pressure repair and blocking endogenous peroxidase treatment, primary antibodies specific for Ki67 (1:300) were added and incubated at 37°C for 60 min, followed by secondary antibodies at 37°C for 30 min. DAB color developer, hematoxylin, alcohol hydrochloride and lithium carbonate solution were added in that order, respectively. Finally, the slices were dehydrated and sealed with neutral gum. The positive area of representative images of macrophages was visualized with an OLYMPUS microscope (CX43, Japan) at 400 magnification and quantitative analysis was performed using ImageJ software (National Institutes of Health, USA). The mean absorbance of the stained areas in each field of view was calculated and finally the mean optical density values of the protein staining in the tissues were determined by comparing the mean values of each group.

2.11 Histopathological staining

Paraffin wax was used to embed a portion of the tissue into wax blocks for sectioning.
Sections were transparently treated by xylene. Subsequently, the sections were washed with water by sequentially eluting the residual xylene with different concentrations of ethanol. Next, the sections were sequentially stained by hematoxylin and eosin, and then dehydrated with high concentrations of ethanol. Finally, the sections were sealed again with the transparency of xylene and then with neutral glue.

### 2.12 Determination of tissue distribution of CP by LC-MS/MS

#### 2.12.1 In vivo analytical method for CP

CP in plasma was determined by LC-MS/MS (AB Sciex QTRAP® 5500, SCIEX, USA). Plasma samples were mixed with the IS (Antipyrine) working solution, three times the volume of plasma methanol was added to precipitate the protein, and after vortex mixing and centrifugation, the supernatant was filtered through a 0.22 μm membrane and fed into the sample. The chromatographic separation system was an ACQUITY UPLC BEH C_{18} column (1.7 μm, 2.1×100 mm) maintained at 30°C. Isocratic elution was carried out with a liquid chromatography mobile phase consisting of 0.1% formic acid in water and chromatographic grade acetonitrile (30: 70; v/v) at a flow rate of 0.2 mL/min and an injection volume of 2 μL. The complete analysis time for one sample takes 4 min. The mass spectrometer was operated in positive electrospray ionisation mode. Detection results were run in multiple reaction monitoring (MRM) mode. The optimized MRM conversion conditions were 261.0→104.1 for CP, 89.8 V for collision voltage (DP) and 29.6 V for fragmentation voltage (CE); 189.2→103.8 for the IS (Antipyrine), 81.1 V for DP and 31.9 V for CE, respectively. The method has been validated according to the FDA guideline for bioanalytical method validation, and the validation method and experimental procedures were referenced to our
previous study (Wang et al., 2019b).

2.12.2 Determination of the tissue distribution of CP

HCC mice were randomly divided into three groups: single injection, repeated injection and KCZ-inhibited groups, and three time points were set for each group for determining the concentration of CP in tissues at different time points, which were 1, 2 and 6 h (n = 3 for each point). The repeated injection group was pre-injected with PEG-B-L (containing 0.05 μmol of PEG2000-DSPE/kg) 3 days ago, while the single injection group was given PBS as a control. KCZ-inhibited group was pretreated by continuous gavage with KCZ (50mg/kg/d) for three days to study the effect of selective CYP3A inhibitors on the tissue distribution of PEG-CP-L prior (Huang et al., 2020). On the third day all animals were injected intravenously with PEG-CP-L (1 mg/kg) and then the tissues were removed according to different time points. The obtained tissue sample was taken 100 mg for each tube, added 5 times the amount of methanol solution containing the internal standard, and put it into a cryogenic grinder for thorough grinding. Then the grinding solution was centrifuged at 10,000 rpm for 10 min, taken the supernatant and repeated the process of high-speed centrifugation, the supernatant could be analyzed according to the conditions of "2.12.1". To calculate the concentration of CP at different time points, the peak areas of CP and IS needed to record.

2.13 Statistics

All data are presented as mean ± standard deviation (SD) of more than three independent experiments. Statistical analyses were performed using SPSS 23.0 software (IBM Corp., Armonk, NY). The significant differences of normally distributed data of multiple comparisons were
analyzed by one-way ANOVA, followed by a Student-Newman-Keuls post-hoc test for statistical comparisons of two groups, and the non-parametric test (Kruskal-Wallis) was applied to detect the data with uneven variance. Statistical differences were defined as $P < 0.05$, $P < 0.01$ and $P < 0.001$.

3. Results

3.1 Characterization of PEG-CP-L

The appearance of PEG-CP-L prepared by the ethanol injection method was light yellow colloidal dispersion system with no insoluble particles visible to the naked eye (Fig. 1A). The results of morphological investigation are shown in Fig. 1B. PEG-CP-L was a unilamellar single-chambered vesicle structure with similar size, regular spherical or sphere-like shape, which did not show adhesion between each other and the structure of hydration layer can be seen. Size and PDI are the most relevant features in liposome characterization. As can be seen from Fig.1C, the average hydrodynamic diameter particle sizes of PEG-B-L and PEG-CP-L were measured by PCS to be $(141.6 \pm 14.40)$ and $(162.2 \pm 13.63)$ nm, both less than 200 nm, which is favorable for the nanomedicine to produce enhanced permeability and retention (EPR) effect in tumor tissues. The PEG-B-L and PEG-CP-L were characterized by the following properties. The PDI values of PEG-B-L and PEG-CP-L were $(0.18 \pm 0.05)$ and $(0.18 \pm 0.05)$, respectively, with low PDI values implying that they are uniformly distributed. Zeta potential is a value used to predict the stability of colloidal systems, and a low Zeta potential means that liposomes are more likely to aggregate over time. Respectively, the Zeta potentials of PEG-B-L and PEG-CP-L were $(-32.6 \pm 2.73)$ and $(-38.8 \pm 2.26)$ mV (Fig. 1D). The encapsulation rate of PEG-CP-L was measured by ultrafiltration centrifugation combined with HPLC with a mean value of $(83.08 \pm 3.11)$. The results of the
characterization of PEG-DiR-L are described in the Supplemental Fig. S1. The average particle size, PDI, and zeta potential of PEG-DiR-L were, respectively, \((156.3 \pm 1.11)\) nm, \((0.24 \pm 0.01)\) and \((-26.0 \pm 0.24)\) mV. The result of measured EE\% was \((97.68 \pm 0.23)\).

3.2 Repeated injection of PEG-L can induce ABC phenomenon in HCC models

Model reliability of rats with HCC was demonstrated in the Supplemental Fig. S2. DEN-induced rats had slower weight gain than normal rats, which may be related to the necrosis of hepatocytes and the induction of liver cancer by DEN (Supplemental Fig. S2A). H&E results showed DEN-treated rats had irregular arrangement of hepatocytes with large nuclei, dark staining, marked cell proliferation, and infiltration of inflammatory cells (Supplemental Fig. S2B). The ALT, ALP and AST of the model group rats were significantly different from those of the normal group rats, which showed that the liver function of the model group rats was impaired and the content of cancer-specific indexes GGT and AFP was increased \((P<0.001)\) (Supplemental Fig. S2C). In conclusion, a reliable primary rat liver cancer model can be obtained after 14 weeks of induction by DEN drug and can be used for subsequent experiments.

Fig. 2A and 2B showed that the concentration as well as the relative blood concentration of DiR in HCC rats pre-induced by PEG-B-L had a faster decreasing trend and elimination rate over 10 h compared with single injection. Additionally, the results of pharmacokinetic parameters in Table 1 showed that the plasma clearance \(CL_z\) and \(V_z\) were significantly higher in the repeated injection group than in the single control group \((P<0.001)\), and \(AUC_{(0 \rightarrow 30 \text{ min})}\), \(AUC_{(0 \rightarrow 10 \text{ h})}\), and \(t_{1/2z}\) in the single injection group were 1.92-fold, 3.63-fold, and 1.74-fold higher than that of the repeated injection group, respectively which were consistent with the results in Fig.2A and 2B. It was suggested that the pre-stimulation of PEG-B-L in HCC rats also accelerated the clearance of
liposomal drugs, which, to the best of our knowledge, has not been investigated in previous reports. In addition to inducing rapid drug clearance in plasma, the ABC phenomenon was accompanied by significant aggregation in the liver and spleen. We then examined the tissue distribution of repeated injections of PEG-L in different species of HCC models. At 1, 2 and 6 h after the last administration, we removed all tissues of HCC rats for fluorescence imaging. DiR was mainly taken up by the liver of HCC rats, and the fluorescence intensity of the liver and spleen of rats in the HCC repeat injection group was significantly higher than that of the single injection group in the same quantitative area within 2 h ($P<$0.001), implying that the drug was significantly aggregated in liver and spleen of HCC rats with repeated injections of PEG-L (Fig. 2C). With the aim of making a more objective evaluation of the ABC phenomenon, we calculated the correlation index of the ABC phenomenon as shown in Table 2. The ABC index was calculated as follows: AUC index = $\frac{\text{AUC}_{(0\rightarrow30 \text{ min})}\text{ of the second dose}}{\text{AUC}_{(0\rightarrow30 \text{ min})}\text{ of the control dose}}$, and an ABC index <1 indicates a greater degree of accelerated blood clearance (Ishihara et al., 2009; Zhao et al., 2012a; Zhao et al., 2012b; Liu et al., 2022a). Hepatic accumulation (HA) index and splenic accumulation (SA) index were used as complements to the assessment of the ABC phenomenon to emphasize the contribution of the liver and spleen. HA index was calculated as follows: HA index = $\frac{\text{Hepatic concentration of repeated group}}{\text{Hepatic concentration of single group}}$. As well, SA index was calculated as follows: SA index = $\frac{\text{Splenic concentration of repeated group}}{\text{Splenic concentration of single group}}$. The higher HA or SA index (> 1) indicates the more increase in hepatic or splenic accumulation, which can lead to the ABC phenomenon (Liu et al., 2022a). The ABC index was (0.53 ± 0.13) for repeated injections, and HA and SA indexes were respectively (2.06 ± 0.29) and (1.83 ± 0.04), confirming that a severe ABC phenomenon exhibited
in the HCC repeated injection group.

Notably, the ABC phenomenon can also occur in situ transplanted HCC mice. As displayed in Fig. 2D, the in vivo fluorescence imaging of HCC mice at different time points after the last administration of PEG-L was performed. Fig. 2E is a semi-quantitative plot of its fluorescence intensity, which showed that the fluorescence intensity was also higher in the repeated injection group than in the single injection group 2 h after injection ($P<0.05$). Similarly, when the tissues were removed for imaging at 6 hours after administration, the fluorescence of the drug was also predominantly concentrated in the liver, and the liver fluorescence was brighter in the repeated injection group than in the single injection group (Supplementary Fig. S3B). It is worth mentioning that our results exclude the effect of free DiR, which was not predominantly distributed in the liver after injection Supplemental Fig. S3A. In conclusion, these data suggest that PEG-L pretreatment triggered a rapid accumulation of the second dose in the liver of HCC rats and HCC mice at the initial time.

3.3 HCC model activation of ABC phenomenon is accompanied by increased activation and expression of CYP3A enzymes

3.3.1 Determination of CYP activity

In this study, the issue of whether repeated injection of PEG-L lead to the activation of CYPs was investigated. For the sake of whether an increase in the activity and expression of CYP3A1 (equivalent to CYP3A4 in humans) also occurs in the HCC rats after induction of the ABC phenomenon. We divided HCC rats into HCC model group, HCC single injection group, HCC repeated injection group and DEX-induced HCC group, and used liver microsomal in vitro incubation method to determine the activity of CYP3A1. 6β-hydroxytestosterone is a metabolite
produced by the conversion of testosterone by CYP3A1, and the testosterone 6β-hydroxylase rate
is commonly used to indicate the activity of CYP3A1. As shown in Fig. 3A, 20 minutes after the
addition of NAPDH to initiate the reaction, the rate of 6β-hydroxytestosterone produced by the
repeated injection group of the HCC microparticle incubation system was 3.33-fold more than that
of the single injection group of the HCC, implying that the conversion of testosterone to
6β-hydroxytestosterone was faster and the CYP3A enzyme activity was higher in this group
\( (P<0.001) \), and which was no significant difference compared with the DEX-induced HCC group.

3.3.2 Determination the expression of CYPs mRNA and protein

RT-qPCR and Western blot results of CYP3A1 in HCC rats showed that mRNA and protein
expression of CYP3A1 were significantly higher in the repeated injection group than in the single
injection group, and that DEX, an inducer of CYP3A1 (Turncliff et al., 2004), further induced the
increase in CYP3A1 expression induced by the repeated injection (Fig. 3B-D). Furthermore, we
divided HCC mice into four groups, namely, the HCC model group, the single injection group, the
repeated injection group, and the KCZ-inhibited HCC group. The results of protein expression and
relative quantitative analysis of CYP3A11 (equivalent to CYP3A4 in humans) in HCC mice are
shown in Fig. 3E and 3F, and Fig. 3G reveals its mRNA results. These data indicate that the
expression of CYP3A11 in the repeatedly injected HCC mice was significantly higher than that of
the single injection group. Meanwhile, KCZ, a mouse CYP3A11 enzyme inhibitor (Gehlhaus et al.,
2007), inhibited the increase in CYP3A11 expression induced by the repeated injection group.

3.4 PEG-L pre-induced delivery enhances the targeted therapeutic effect of PEG-CTX-L in HCC

Based on the fact that the HCC model can stimulate the ABC phenomenon with hepatic
accumulation and CYP3A induction, which encouraged us to further explore the potential
application of ABC phenomenon. We selected an anticancer prodrug, CP, which requires to undergo oxidative activation by CYP3A, prepared it as PEG-L to enhance the hepatic targeting of the drug using the ABC phenomenon and to accelerate the conversion of the prodrug in the liver. HCC mice were divided into four groups (n=8 for each group), and Table 3 presents the specific administration of one cycle. After four cycles of drug administration, the mice were anesthetized with 2.5% isoflurane and then plasma was obtained by centrifugation of blood collected from the posterior venous plexus of the eye. All organs of the mice were also removed, part of which were fixed in formalin for pharmacodynamic analysis, and part of which were stored in the refrigerator at -80°C.

The changes in body weight of mice during drug administration are shown in Fig. 4A. Except for the CP administration group, the rest of the administration methods had no significant effect on the body weights of the mice, indicating that the administration of PEG-L was well biocompatible, and the body weights of the mice in the CP group decreased slightly at the later stage, which might be related to the pharmacological toxicity of CP. The liver organ index of the repeated injection group was significantly lower than that of the single administration group (Fig. 4B). The tumors were circled with red circles as shown in Fig. 4C, and the volume of the tumors was shown in Fig. 4D, which indicated that the administration method of the repeated injection group could effectively reduce the size and weight of the tumors, and has a good effect on tumor growth inhibition. The results of H&E staining and immunohistochemical staining for Ki67 were used to analyze the apoptosis and proliferation inhibition of tumor cells after treatment (Fig. 4E). As a result, compared with the single-injection group, the area of tumors that did not show obvious necrosis in the repeated injection group was significantly reduced, and the number of...
Ki67-positive cells with the lowest number of expressions. AST, AKP, and ALT, which were the commonly used indicators for measuring the liver function, in the repeated injection group were significantly lower than that of the single injection group \( (P<0.001) \) (Fig. 4G-I). The liver function indexes in the injection group were significantly lower than those in the single injection group. Although CP is clinically known for its high organ toxicity, this method of administration did not cause significant toxicity to other organs based on H&E staining of the other major organs in mice (Luo et al., 2023), probably because the 5 mg/mL dose given once every 6 days did not reach the damaging dose (Supplemental Fig. S4).

3.5 ABC phenomenon induces intrahepatic accumulation of PEGylated anticancer prodrug liposomes and produces favorable anticancer effects in dependence on CYP3A enzymes

To explore the pharmacokinetic mechanism of therapeutic effect of repeated injections of PEG-L for HCC, we used the CYP3A enzyme inhibitor KCZ (50 mg/kg/d) by gavage for 3 consecutive days prior to the repeated administration (Huang et al., 2020). The same dose of CMC-Na was given to the control group, and the rest of the administration regimen was the same as that in Table 3. Tumor volumes of the HCC mice in the CYP3A enzyme inhibitors group were significantly larger than that of the control group (Fig. 5A and 5B), and the indexes of ALT, AST and AKP were significantly higher than that of the control group (Fig. 5C), implying that CYP3A enzyme inhibition led to the weakening of the anticancer effect, which might be related to the reduction of the accumulation of the anticancer active ingredients in the tumor site due to the reduction of the effective conversion rate of CP. Thus, we analyzed the expression of CYP3A11 protein at the tumor site in the CMC-Na control and inhibitor groups, as shown in Fig. 5D and 5E. The results showed that the expression content of CYP3A enzyme in the inhibitor group was
significantly lower than that in the control group ($P<0.001$). Moreover, by using LC-MS/MS technique, CP content in the liver and spleen sites of different groups of HCC rats were analyzed. The results of the concentration and relative concentration of CP in the liver at different time points are shown in Fig. 5F and 5G, respectively. The concentration of hepatic CP in the CMC-Na control group was significantly higher than that in the KCZ-inhibited group, and the elimination of CP was significantly faster than that in the KCZ-inhibited group, which suggests that the inhibition of CYP3A activity suppressed the hepatic accumulation of CP to a certain degree, indicating that the activation of CYP3A is involved in the phenomenon of ABC, which may affect the conversion of CYP3A-activated prodrugs. Notably, CP levels in the KCZ-inhibited group were significantly higher than those in the single injection group at 1 h ($P<0.05$), revealing that CP can also accumulate in the liver with reduced CYP3A activity, albeit to a lesser extent than in the CMC-Na control group. The results of splenic CP concentration are demonstrated in Supplemental Fig. S5, and at 1 h, the splenic CP concentration was significantly higher in the CMC-Na group than in the KCZ-inhibited group. In conclusion, the activation of ABC phenomenon by pre-injection of PEG-L induced the hepatic accumulation of the second dose of PEG-CP-L, which was accompanied by the activation of hepatic CYP3A enzyme and the increase of its expression, which could accelerate the conversion of CP into a cytotoxic drug to exert a better anti-hepatocellular carcinoma effect.

4. Discussion

Most of the previous studies on the ABC phenomenon have utilized normal models to explore the mechanism of the ABC phenomenon (Ishida et al., 2006b; Koide et al., 2010; Shimizu et al., 2015; Liu et al., 2022a) or centered on how to eliminate the ABC phenomenon (Li et al., 2013;
Wang et al., 2017; Abu-Dief et al., 2022; Liu et al., 2022b), with few reports on pathological models. Since resistance and hypersensitivity to chemotherapeutic agents in HCC patients lead to poor treatment outcomes, it appears interesting to verify whether the ABC phenomenon can still be generated in HCC models and the impact of the underlying ABC phenomenon on the efficacy potentially of HCC. Our data indicated that after stimulation by pre-injection of PEG-L, both HCC rats and mice showed significant aggregation of second-injected PEG-L in the liver and spleen, and the second dose of PEG-L was rapidly cleared from the plasma of HCC rats (Fig. 2). Furthermore, the combined use of ABC index and HA/SA index allows for a more comprehensive determination of the occurrence of the ABC phenomenon, and at the same time corresponds to the most distinctive features of the ABC phenomenon: rapid clearance of the drug from the plasma significant aggregation of the drug in the liver and the spleen (Table 2). To the best of our knowledge, this is the first time that the HCC model has been demonstrated in a study to also induce the ABC phenomenon. HCC is considered as a model of inflammation-derived cancer caused by chronic liver injury, which contains a large number of immune cells, especially KCs, which can not be found anywhere else in the body (Yuen and Wong, 2020; Giraud et al., 2021; Li et al., 2021). In view of the fact that KCs ingested the immune complex of PEG-L and anti-PEG-antibody injected twice (El Sayed et al., 2020; Kozma et al., 2020), we speculative that the strong immune system stimulated in patients with liver cancer may lead to the occurrence of ABC phenomenon.

Many studies have shown that hepatic CYP450 enzyme activity and expression are significantly lower in hepatocellular carcinoma patients than in paracancerous tissues (Yu et al., 2018; Lauschke et al., 2019; Flannery et al., 2020), which hinders the therapeutic effects of anticancer
pre-drugs that require oxidative and reductive activation by CYP450. In addition, the toxic side effects caused by poor targeting of anticancer prodrugs are also a challenge to be solved. It is worth mentioning that our study published in DMD showed that the activity and expression of CYP450 enzymes were affected by the induction of the ABC phenomenon, in particular CYP3A. This prompted us to explore whether the induction of the ABC phenomenon in the HCC model would activate CYP3A and upregulate CYP3A expression. To elucidate this issue, we used in vitro incubation with liver microsomes to assess CYP3A enzymatic activity. These results suggested the testosterone 6β-hydroxylase rate in HCC rats with repeated injections of PEG-L was significantly higher than that of the single injection group, and this phenomenon could be enhanced by the CYP3A enzyme inducer DEX, which indicated that the CYP3A enzyme activity was higher in the repeated injection group. Meanwhile, the results of WB and RT-qPCR verified that the expression of CYP3A enzyme in the repeated injection group was significantly increased (Fig. 3A-D). Given that mouse CYP3A11 has the highest similarity to human CYP3A4, mice were also chosen to study the metabolic profiles of CYP3A substrates (Paine et al., 1997; Martignoni et al., 2006). Similarly, repeated injections of HCC mice were shown to significantly upregulate CYP3A enzyme expression, and the rodent CYP3A enzyme inhibitor KCZ reversed the effects of the ABC phenomenon (Fig. 3E-G).

In the context of solid tumors, the EPR effect has become an important driver of cancer nanomedicine design. In order to meet the design requirements of tumor nanodrugs, we controlled the PEG-CP-L prepared by ethanol injection method to have a particle size of less than 200 nm, an absolute value of the Zeta potential of more than 30 mV, a unicompartmental vesicle structure, and a high EE% (Fig. 1). However, in recent decades, the EPR effect has been highly controversial.
Due to the complexity of the tumor environment, the EPR effect is highly variable, and a number of adverse reactions arising in the clinical setting have led to its questioning, and passive targeting solely relying on the EPR is unable to satisfy the requirement of effective anticancer treatment, which is urgently needed to be complemented by other targeting strategies (Shi et al., 2020; Ikeda-Imafuku et al., 2022; Sharifi et al., 2022). Since HCC mice can induce ABC phenomenon by inducing PEG-L accumulation in the liver and CYP3A enzyme activation, we pinned our hopes on selecting an anticancer prodrug that needs to undergo CYP3A enzyme activation and preparing it into liposomes, which can exert a targeting effect on hepatocellular carcinoma by inducing ABC phenomenon. The therapeutic effect of repeated injections of PEG-L on HCC was assessed by examining the body weight changes, hepatic organ index, volume of solid tumors and related factors in mice. As mentioned above, PEG-L preinduction could effectively reduce the volume of solid tumors and decrease apoptosis, which could stimulate the antitumor effect of a single injection of PEG-CP-L without toxicity to other organs (Fig. 4 and Supplemental Fig. S4). For the purpose of further elucidating the pharmacokinetic mechanism of the administration of PEG-L repeated injections delivering prodrugs for the treatment of HCC mice. We utilized CYP3A enzyme inhibitors to explore the triple relationship between CYP3A enzyme expression level, metabolic concentration of the prodrug, and potency of the drug. Our results were found that despite the accelerated hepatic uptake of the prodrug after induction of the ABC phenomenon, the conversion process of CP was inhibited due to the suppression of CYP3A expression and activity in the CYP3A enzyme inhibitor group, resulting in a significantly larger solid tumor volume in the CYP3A inhibitor group than in the CMC-Na control group. This indicates that the increase in hepatic accumulation and CYP3A enzyme expression is a key mechanism in the treatment of HCC
by the repeated injection PEG-L delivery strategy (Fig. 5).

Although these studies reveal important findings, there are some limitations. Firstly, the CYP3A11-specific inhibitor KCZ is of limited value, and the results would have been more convincing if CYP3a11-specific adenoviral short-interfering RNA molecules could have been used to inhibit CYP3A expression and activity, respectively. Secondly, mouse CYP3A11 is a homologue of human CYP3A4 and is thought to have a similar function to CYP3A4, but some studies have shown limitations in the study of CYP3A4 regulation in wild-type mice and humans, therefore, humanized CYP3A4 mice would better validate our experimental results (Crosby and Riddick, 2019). The study on the mechanism of action of repeated injections of PEG-L for the treatment of HCC did not analyze the CYP3A enzyme metabolites of CP and was limited to the expression of CYP3A and the hepatic accumulation of CP, which lacked more in-depth studies, and we believe that more in-depth studies will be conducted in the future. It is worth noting that proper in vivo validation is required to apply this therapeutic approach to the clinical arena, as well as following strict dosing principles, i.e., secondary injections of PEGylated cytotoxic liposomal drugs usually cannot be induced by the first dose of PEGylated cytotoxic liposomes to produce the ABC phenomenon, e.g., repeated injections of PEGylated adriamycin liposomes do not trigger ABC phenomena related to the fact that adriamycin disrupts the secretion of anti-PEG-antibodies by splenic B cells (Ishida et al., 2006a; Suzuki et al., 2012). Moreover, to ensure that hepatic aggregation and CYP3A enzyme activation can be induced, repeat injections are generally preferred for 3-7 days (Li et al., 2013; Saadati et al., 2013).

Taken together, repeated intravenous injection of PEG-L was found to induce ABC phenomenon in HCC rats and HCC mice, and this induced ABC phenomenon activated CYP3A
enzymes and upregulated their expression. Repeated injection of PEG-L can increase the delivery of CP, an anticancer prodrug, to the liver and accelerate the effective conversion of CP, which is a highly effective and low-toxicity strategy for the targeted treatment of HCC. This study is characterized by the use of the phenomenon unique to PEG-L to achieve a win-win situation for simple agents, reducing the toxicity of anticancer pro-drugs through liver targeting and enhancing the anti-tumor effect through the enhancement of enzyme activity and expression, which breaks the stereotypical impression of the ABC phenomenon and provides a reliable experimental basis for the further development of the PEG-L drug delivery system for the clinical treatment of HCC.

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Data Availability Statement

The authors declare that all the data supporting the findings of this study are available within the paper and its Supplemental Data.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author contributions

Participated in research design: F.L. Wang, Y.N. Chen, and W.D. Chen.

Conducted experiments: Zhang and Pan.

Contributed new reagents or analytic tools: L. Wang, Ye, L and Meng.
Performed data analysis: F.L. Wang and Zhang.

Wrote or contributed to the writing of the manuscript: Zhang and F.L. Wang.

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**Footnotes**

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**Legends to the figures**

**Fig. 1.** Characterization of PEG-CP-L. (A) Photograph of PEG-CP-L solution. (B) Morphology of PEG-CP-L measured by a transmission electron microscope (TEM). Scale bar represents 200 nm. (C) Size distribution and (D) Zeta potential of PEG-CP-L and PEG-B-L measured by photon correlation spectroscopy (PCS) (mean ± SD, n = 3).

**Fig. 2.** Effects of repeated injections of PEG-L on HCC patterns. (A) Influences of repeated injections of PEG-L on plasma pharmacokinetics in HCC rats. Plot of drug-time profile and relative drug-time profile of DiR in plasma of HCC rats with repeated injections of PEG-L. (B) Relative drug-time curves were plotted as the ratio of the mean drug concentration at the first time point of the single injection group as 100% and the concentration at the remaining time points to it (mean ± SD, n = 6). (C) Tissue distribution and semi-quantitative ROI analysis at 1, 2 and 6 h after injection of PEG-DiR-L in the plasma of HCC rats repeatedly injected with PEG-L (mean ± SD, n
In vivo imaging and (E) semi-quantitative ROI analysis at 1 h, 2 h and 6 h after injection of HCC mice repeatedly injected with PEG-L at different time points (mean ± SD, n = 3, ***P < 0.001, **P < 0.01, *P < 0.05).

**Fig. 3.** Activity and expression of CYP3A in liver tissues of HCC model with repeated injections. (A) Testosterone 6β-hydroxylase rate of liver microsomes in rats with repeated injections HCC rats co-incubated with testosterone. (B) Hepatic CYP3A protein expression and (C) relative protein quantification in repeatedly injected HCC rats. (D) mRNA results of hepatic CYP3A in repeatedly injected HCC rats. (E) Hepatic CYP3A protein expression and (F) relative protein quantification in repeated injection group. (G) mRNA results of hepatic CYP3A in repeated injection group (n = 3, mean ± SD, ***P < 0.001, **P < 0.01, *P < 0.05).

**Fig. 4.** Efficacy of repeated injections as a mode of administration for the treatment of HCC. (A) Body weight changes in HCC mice during pharmacodynamic drug administration. (B) Hepatic index in HCC mice. Hepatic index = Hepatic weight (g) / Body weight (100 g). (C) Photograph of liver tumors in HCC mice. (D) Tumor volume in HCC mice. Tumor volume = 1/2 ab² (a is the long diameter, b is the short diameter). (E) Histopathologic staining and Ki67 immunohistochemical findings in the liver. (F) Ki67 positive cell rate and (G-I) serum biochemical indices determined by the microplate method. Data are presented as the mean ± standard deviation (n = 7, ***P < 0.001).

**Fig. 5.** Pharmacokinetic mechanism of repeated injections of PEG-L for the treatment of HCC. (A) Effect of CYP3A enzyme inhibitors on tumor size of repeated injections of PEG-L. (B) Effect of CYP3A enzyme inhibitors on tumor volume of repeated injections of PEG-L (n = 7, mean ± SD, ***P < 0.001, compared with CMC-Na control group). (C) Effect of CYP3A enzyme inhibitors on
serum biochemical indices of repeated injections of PEG-L administration (n = 7, mean ± SD, ***P
<0.001, *P<0.05, compared with CMC-Na control group). (D) Protein expression and (E)
relative protein quantification of CYP3A enzyme by CYP3A enzyme inhibitors in repeated
injections of PEG-L administration (n = 3, mean ± SD, ***P <0.001, compared with CMC-Na
control group; #P <0.05, compared with HCC single group). (F) Effect of CYP3A enzyme
inhibitors on the concentration and (G) relative concentration of hepatic CP administered by
repeated injections of PEG-L (n = 3, mean ± SD, ***P <0.001, *P<0.05, compared with
CMC-Na control group; #P <0.05, compared with HCC single group).

Tables

**Table 1** Pharmacokinetic parameters of CP in HCC rats after repeated injections (mean ± SD, n =

<table>
<thead>
<tr>
<th>Pharmacokinetic parameters</th>
<th>units</th>
<th>HCC single injection</th>
<th>HCC repeated injection</th>
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<tbody>
<tr>
<td>AUC(0→30 min)</td>
<td>µg/L·h</td>
<td>222.40 ± 32.91</td>
<td>115.79 ± 22.48***</td>
</tr>
<tr>
<td>AUC(0→10 h)</td>
<td>µg/L·h</td>
<td>2733.62 ± 344.44</td>
<td>753.83 ± 131.71***</td>
</tr>
<tr>
<td>MRT(0→10 h)</td>
<td>h</td>
<td>3.814 ± 0.17</td>
<td>2.89 ± 0.19***</td>
</tr>
<tr>
<td>t1/2z</td>
<td>h</td>
<td>5.72 ± 1.01</td>
<td>3.09 ± 0.32***</td>
</tr>
<tr>
<td>CLz</td>
<td>L/h/kg</td>
<td>2.14 ± 0.24</td>
<td>1.23 ± 0.19***</td>
</tr>
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</table>
Abbreviations: AUC$_{(0\rightarrow30\text{ min})}$, area under the plasma concentration-time curve from time 0 to 30 min time point; AUC$_{(0\rightarrow10\text{ h})}$, area under the plasma concentration-time curve from time 0 to 10 h time point; t$_{1/2}$, half-life; CP, Cyclophosphamide; CLz, plasma clearance; MRT$_{(0\rightarrow10\text{ h})}$, mean residence time from time 0 to 10 h time point; SD: standard deviation; ***$P<0.001$, **$P<0.01$, *$P<0.05$, compared with HCC single injection group.

Table 2 HCC rats for the ABC phenomenon of ABC index, HA index and SA index (mean ± SD, n = 6)

<table>
<thead>
<tr>
<th>Indexs</th>
<th>Single injection</th>
<th>Repeated injection</th>
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<tbody>
<tr>
<td>ABC index</td>
<td>1.00 ± 0.00</td>
<td>0.53 ± 0.13</td>
</tr>
<tr>
<td>HA index</td>
<td>1.00 ± 0.00</td>
<td>2.06 ± 0.29</td>
</tr>
<tr>
<td>SA index</td>
<td>1.00 ± 0.00</td>
<td>1.83 ± 0.04</td>
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The ABC index was calculated as follows: ABC index = AUC$_{(0\rightarrow30\text{ min})}$ of repeated group / AUC$_{(0\rightarrow30\text{ min})}$ of single group. HA index was calculated as follows: HA index = Hepatic concentration of repeated group / Hepatic concentration of single group. SA index was calculated as follows: SA index = Splenic concentration of repeated group / Splenic concentration of single group. Data are mean ± standard deviation of three repeats. Abbreviations: ABC, accelerated blood clearance; HA, hepatic accumulation; SA, splenic accumulation.

Table 3 Pharmacodynamic dosing regimen for one dosing cycle

<table>
<thead>
<tr>
<th>Group</th>
<th>Initial injection</th>
<th>Interval time (d)</th>
<th>second injection</th>
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<tr>
<td>Modle control</td>
<td>saline</td>
<td>3</td>
<td>saline</td>
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<tr>
<td>CP</td>
<td>saline</td>
<td>3</td>
<td>CP</td>
</tr>
<tr>
<td>Single dose</td>
<td>saline</td>
<td>3</td>
<td>PEG-CP-L</td>
</tr>
<tr>
<td>Repeat dose</td>
<td>PEG-B-L</td>
<td>3</td>
<td>PEG-CP-L</td>
</tr>
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</table>

A dosing cycle is divided into two administrations separated by three days. The first dose of PEG-B-L was 0.05 μmoL of PEG2000-DSPE/kg and the second injection was 5 mg/kg.

Abbreviations: CP, Cyclophosphamide; PEG-B-L, blank PEGylated liposomes; PEG-CP-L, PEGylated liposomal Cyclophosphamide.
Figure 5

A

B

C

D

E

F

G