Supplementary Information

In vitro and in vivo evaluation of PEG-conjugated ketal-based chitosan micelles as the pH-sensitive carriers

Daquan Chen*, and Jingfang Sun

School of Pharmacy, Yantai University, 30 Qingquan Road, Laishan District, Yantai 264005, China

* Correspondence: Daquan Chen, School of Pharmacy, Yantai University, 30 Qingquan Road, Yantai, 264005, People's Republic of China.

E-mail address: cdq1981@126.com(Chen).

[1] Both authors contributed to this work equally.

Tel. +86 535 6706021

Fax +86 535 6706021

Experimental section

Materials

Curcumin (Cur) was supplied by Yantai Science & Biotechnology Co. Ltd. (China). Chitosan was provided by the Shanghai Lanji Co. Ltd., China with deacetylation degrees of 97% and viscosity average molecular weight of 65,000 D. mPEG2000, 1Ethyl3(3dimethyllaminopropyl) (EDC), 4-(dimethyl amino) pyridine (DMAP), and Succinic anhydride were obtained from Sigma Chemical Co. (MO, USA).MTT was obtained from Sigma Chemical Co. (MO, USA). All other reagents were of analytical

grade and supplied by Sinopharm Group Chemical Reagent Corp.

Preparation and characterization of PCK polymer

The PCK polymer was synthesized as described in our previous report [30]. In brief, the product mPEG2000-chitosan (mPEG-CS) was obtained by mPEGCHO (2000 D) NaCNBH3 with amino group of chitosan. Thereafter, the active carboxyl of IPGSA was conjugated to the backbone of mPEG-CS in the presence of EDC and DMAP. The molar ratio of PEG group and ketal group moiety was about 6:1. A white PCK powder was obtained. The structure of the PCK polymer was determined by FT-IR and ^{1}H-NMR. [30].

Preparation and characterization of the Cur-PCK micelles

The pH-sensitive Cur-loaded PCK micelles (Cur-PCK) was prepared by the enhanced thin-film hydration method. Briefly, the pH-sensitive PCK and curcumin (3:1) was dissolved in chloroform. The organic phase was removed at 40 ° C on a rotary flask. The flask was evaporated under reduced pressure. The dry lipid formed was hydrated with phosphate buffer saline (pH 7.4). The Cur-PCK suspension was filtered through 0.2 mm polycarbonate filters and stored at 4 ° C until use. The particle size, size distribution and zeta potential of Cur-PCK were determined by dynamic light scattering (DLS). The morphology was observed by atomic force microscopy (AFM,Nano Scope IIIa, Veeco, USA). The enhanced method of entrapment efficiency (EE) was also tested under the previous study.

In vitro cellular uptake and of Cur-PCK micelles

In vitro cellular uptake and intracellular behavior of Cur-PCK micelles was assayed by the confocal laser scanning microscopy (CLSM). As a novelty, curcumin (Cur), was incorporated into PCK micelles not only as a theraputic drug but also a fluorescence marker. The Cur was dissolved in ethanol as the control (5 mg/mL). MCF-7 cells were seeded at a density of 10^5 cells/well. After 24 h, the medium was added by Cur-PCK micelles diluted in the medium (5 mg/mL). At the different time points (0.5, 1, 2, 4, 6h after incubation), the cells were washed three times with PBS

and fixed with 4% paraformaldehyde for 15 min. Finally, the cells were observed using Olympus Flowview FV 1000 (Ex 488 nm).

Ex vivo imaging of Cur-PCK micelles

The studies of ex vivo imaging of Cur-PCK micelles with the nearinfrared fluorescence (NIRF) dye (DiR) as the marker, were tested with noninvasive optical imaging system [31]. The Kunming mice (n=10) were injected with free DiR (48 ug/ml) as the control and DiR labeled PCK micelles (48 μg/ml) by the tail vein. The imaging tests were carried out at 10min, 30min, 1 h, 5 h, 8 h, 12 and 24 h post-injection using a Carestream Molecμlar Imaging FX PRO (Carestream Health, Inc., USA) equipped with an excitation bandpass filter at 720 nm and an emission at 790 nm. Images were analyzed using the Molecular Imaging Software 5.X.

Pharmacokinetic Studies of Cur-PCK micelles

The pharmacokinetic studies of Cur-PCK micelles with SD rats (220-260 g) were tested and intravenously administrated by the tail vein. Rats were randomly divided into the following two groups (n=5): (1) Cur-PCK micelles (10 mg/kg); (2) Cur dissolved in DMSO as the control (10 mg/kg). The blood samples (0.5 mL) were obtained from the plexus venous in the eyeground at 5 min, 15 min, 30 min, 45min, 1 h, 2 h, 4 h, 6 h, 8 h, 12 h and 24 h. The plasma were collected by centrifugation at 5000 rpm for 10 min and stored at −20 ° C. 100 μL of blood sample, and 200 μL of acetonitrile were added into a 1.5 ml protein precipitation tube. The mixture was vortexed for 3 min and centrifugated at 12000 rpm for 10 min. The volume of the sample injected was 10 μL. The mobile phase was a mixture of acetonitrile and 4% (w/v) of acetic acid at the volume ratio of 60:40(V/V). The flow rate was set at 1.0 mL/min and the wavelength was at 425 nm. The column was a Inertsil® ODS-SP (4.6×250 mm, 5 μm), the column temperature was 25°C.

Tissue Distribution Studies of Cur-PCK micelles

Tissue Distribution Studies of Cur-PCK micelles was carried out with Kunming mice (18–22 g). Two formulations (Cur in DMSO suspension and Cur-PCK) with the dose of 10 mg/kg were administrated by intravenous After 0.17, 0.33,0.5, 1, 2, 4, 6, 8,

12, and 24 h of injection, blood samples were obtained from the eyes in each group(n=6).After sacrificed by cervical dislocation, the blood was immediately treated as described in Section 2.6. The organs (heart, liver, spleen, lung, and kidney) were removed and washed twice with 0.9% NaCl, weighed, and homogenized. 100 μL of homogenate and 100 μL of acetonitrile were added and vortexed for 3 min, then centrifugated at 15,000 rpm for 10 min. 10 μL of the supernatant was injected onto the HPLC system as shown in Section 2.6. The parameters of blood, heart, liver, spleen, lung, and kidney were tested by the statistical moment method.

In Vivo Tumor Efficacy Study of Cur-PCK micelles

In our previous study, the primary anti-tumor efficacy of Cur-PCK micelles in vivo was evaluated in tumor-bearing BALB/c mice. The results indicated that the tumor volume of the saline control group was excessively enlarged (> 1000 mm^3), while the other groups were much smaller. The Cur-PCK group suppressed tumor growth most efficiently. The further anti-tumor efficacy of Cur-PCK micelles was evaluated. The tumors were excised for pathology study.

Statistical analysis

All the data were presented as mean±SD from three to ten independent measurements in separate independent experiments and analyzed using descriptive statistic and single-factor analysis of variance.

.