Supporting information

Materials and Measurements

10-HydroxyCamptothecin (HCPT) was purchased from Knowshine (Shanghai, China), and ethanol from Sinopharm Chemical Reagent Co. (China). Fetal bovine serum (FBS), Roswell Park Memorial Institute-1640 (RPMI-1640) medium, FA-free RPMI-1640 and Penicillin-streptomycin solution were purchased from Invitrogen (San Diego, CA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma Aldrich (Milwaukee, WI). Distilled water was obtained from a Milli-Q Biocel water purification system (18.2 MΩ·cm resistivity) (Millipore Corporation, Breford, USA). A human nasopharyngeal epidermal carcinoma cell line (KB cell) was provided by American Type Culture Collection (ATCC). UV-vis absorption spectra were obtained on a Perkin-Elmer Lambda 750 UV/vis/NIR absorbance spectroscopy. The fluorescence spectra were obtained from a FluoroMax 4 (Horiba Jobin Yvon) spectrofluorimeter. Scanning electron microscopic (SEM) images were obtained on a FEI Quanta 200 FEG field emission scanning electron microscope operated at an accelerating voltage of 30 kV. Transmission electron microscopy (TEM) images were taken from FEI Tecnai G2 F20 S-TWIN operated at an accelerating voltage of 200 kV. Dynamic light scattering (DLS) measurements were performed on a Zetasizer Nano ZS (Malvern Instruments, Malvern, U.K.) with a 633 nm He-Ne laser.

Preparation and Functionalization of HCPT NPs

HCPT NPs were prepared by a solvent exchanging method. Briefly, 300 μL of 1×10^{-3} M HCPT/ethanol solution was poured into 5 mL of aqueous solution at 50 °C under vigorous stirring at 1000 rpm. After mixing for 5 min, the sample stabilized for 72 hours. C18PMH-PEG-FA was synthesized following a literature procedure. 1 mg C18PMH-PEG-FA polymer was dispersed completely in 10 mL distilled water for further use. For functionalization of HCPT NPs, 300 μL of C18PMH-PEG-FA/H_2O was added to 5 mL of HCPT NPs suspensions, and the mixture was then under ultrasonic treatment for 5 min. Afterwards, amphipathic polymers C18PMH-PEG-FA would be readily anchored to the surface of HCPT NPs by noncovalent hydrophobic interaction.

Characterization of HCPT NPs and Functionalized HCPT NPs

The morphology and particle size were characterized by SEM and TEM. Samples for SEM were prepared by placing a few drops of sample solutions onto a silicon substrate and then dried in air. Before SEM examination, 2 nm layer of Au was deposited on samples. For TEM analysis, a few drops of the sample solution were placed on a carbon-coated copper TEM grid and dried in air before observation. The nanoparticles size was further measured by DLS at 25 °C. The data reported represented an average of five measurements with ten scans each. The stability of the functionalized HCPT NPs in physiological saline was also investigated by using DLS.

HCPT Loading and Release from Functionalized HCPT NPs

The drug loading content (DLC) was defined as the weight ratio of the encapsulated drugs to the entire drug-loaded nanomedicines. The concentration of functionalized HCPT NPs was determined by UV-vis measurement at 382 nm from a mixture of ethanol and HCPT NPs suspensions (v/v = 9:1), which is about 60 μmol/L (Figure below). The volume of suspensions is 5 mL, thus the weight of encapsulated drugs is $W_{HCPT} = C \times V \times M = 60\ \mu mol/L \times 5 \times 10^{-3}\ L \times 364.35\ g/mol = 1.09305 \times 10^{-4}$ g. The weight of the entire drug-loaded nanomedicines is the total weight of drugs (W_{HCPT}) and the added surfactants ($W_{surfactant}$). Here, 300 μL of 0.1 mg/mL C18PMH-PEG-FA/H_2O was used, the weight is $W_{surfactant} = C \times V \times M = 0.1\ mg/mL \times 300 \times 10^{-3}$ mL $= 3 \times 10^{-5}$ g. Thus, it is estimated that DLC (wt. %) = $W_{HCPT}/ (W_{HCPT} + W_{surfactant}) = 1.09305 \times 10^{-4}/(1.09305 \times 10^{-4} + 3 \times 10^{-5}) = 78\%$.

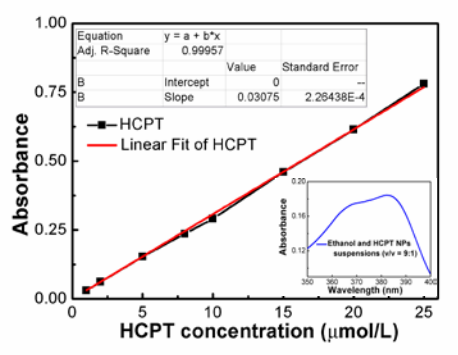

Figure. The absorbance of HCPT molecules at 382 nm (from a mixture of ethanol and water (v/v = 9:1)) as a function of HCPT concentration. Inset: UV-vis absorbance spectra of ethanol and HCPT NPs suspensions (v/v = 9:1).

The release behaviors of HCPT from the functionalized HCPT NPs were studied in phosphate-buffered saline (PBS, pH 7.4) using dialysis tubes at 37 °C. Functionalized HCPT NPs suspensions (2 mL) were added into a dialysis bags (MWCO 5000) and immersed into 50 mL of PBS with stirring for drug release. Aliquots of 2 mL were at a predetermined time withdrawn from the solution to containers. The solution volume was kept constant by adding 2 mL fresh PBS after each sampling. The amount of HCPT released was determined by using fluorescence measurement (excitation at 382 nm). The drug release assay was carried out three times and the average value of the three measurements was taken.

Cell Culture

KB cells were cultured in normal RPMI-1640 and FA-free RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% (v/v) penicillin/streptomycin solution at 37 °C in a humidified atmosphere containing 5% CO_2. The concentration of FA in serum-containing FA-free medium is only 3 nM, as opposed to 2.26 μM under normal culture conditions. Cells were routinely passaged by treatment with trypsin (0.05%)/EDTA.

Cytotoxicity Assays

The *in vitro* cytotoxicity was measured using a standard MTT assay. KB cells were seeded into 96-well plates (100 μL/well). The plates were then incubated at 37 °C for 24 h in a humidified atmosphere containing 5% CO_2. Cells were then treated with various concentrations of free HCPT, HCPT NPs or FA-HCPT-NPs. The free HCPT was usually first dissolved in DMSO to form drug solutions with various concentrations, which were then treated with cells. The drug-treated cells were incubated at 37 °C for pre-determined time in a humidified atmosphere containing 5% CO_2. The cells were then treated with 20 μL of MTT solution (5 mg/mL in PBS) and incubated for 5 h. The medium was removed and the cells were lysed by adding 150 μL of DMSO, the cell viabilities were then measured by MTT assay and the relative cell survival percentages compared to the drug-free control were plotted against the drug concentration in logarithmic scale. For the FA targeted HCPT delivery experiment, KB cells cultured in FA free RPMI-1640 medium with high FR expression were used as positive cells, while KB cells cultured in normal RPMI-1640 medium with low FR expression were used as the negative control. Both positive KB cells and negative KB cells were incubated with a series of concentrations of HCPT NPs, and FA- HCPT NPs. The drug-treated cells were then further incubated for 8 h in a humidified atmosphere containing 5% CO_2 at 37 °C, after which the drug-containing media were removed by aspiration. The remaining cell layers were washed with PBS buffer (2 × 150 μL) followed by replacement with fresh growth media (100 μL). The plates were then maintained in the incubator at 37 °C for a further 16 and 40 h, the cell viability was then measured by MTT assay. The dose-response curves were obtained by using Origin 8.0 and the half-maximal inhibitory concentration (IC50)

values were determined on the basis of the sigmoidal logistic fitting data.

Confocal imaging of cells

Confocal imaging of cells was performed using a Leica laser scanning confocal microscope. Imaging of HCPT was carried out under 405 nm laser excitation and emission was collected in the range of 500 nm to 600 nm. For FA targeted cell imaging, both positive and negative KB cells were incubated with HCPT NPs or FA- HCPT NPs for 2 h at 37 °C before imaging. The concentrations of HCPT was 6 μM in all samples. All cells were washed twice with PBS buffer before confocal imaging. Obtained confocal images were converted to TIFF format by using ZEN 2007 Light Edition SP1 software (Carl Zeiss). All images were taken under the identical instrumental conditions and presented at the same intensity scale.

Cellular uptake measured by fluorescence measurement

KB cells cultured in FA free RPMI-1640 medium with high FR expression [FR(+)] were used as positive cells. FR(+) KB cells were seeded in 24-well plates (1 mL/well). The plates were then incubated at 37 °C for 24 h in a humidified atmosphere containing 5% CO_2. The cells were then incubated with HCPT NPs and FA-HCPT NPs. The drug-treated cells were incubate d for 2 h at 37 °C, and then washed twice with cold PBS, and digested by trypsin (0.05%)/EDTA treatment. The suspensions were centrifuged at 1000 rpm and 4 °C for 4 min. The supernatants were discarded and the cell pellets were washed with PBS to remove the background fluorescence in the medium. After two cycles of washing and centrifugation, cells were resuspended with 2 mL PBS and disrupted by vigorous sonication. The amount of HCPT in sonicated mixture was analyzed by using fluorescence measurement (excitation at 382 nm).

Supporting Figures

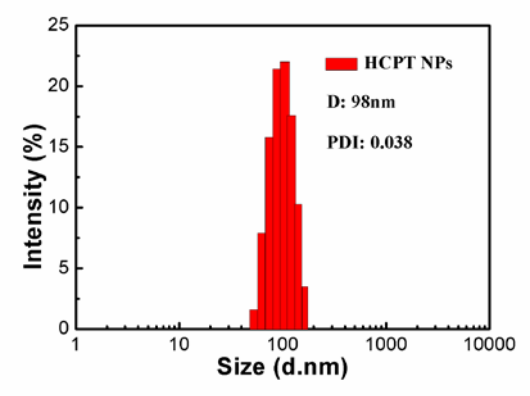

Fig. S1 Size distribution of the particles by DLS measurements at 25 °C in water.

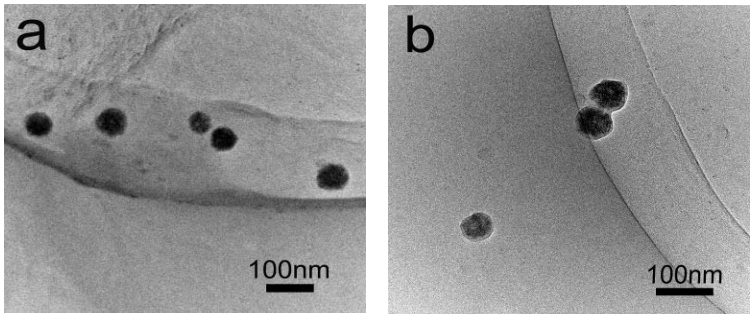

Fig. S2 (a) TEM images of the HCPT NPs and (b) of the functionalized HCPT NPs. Scale bars: (a) 100 nm and (b) 100 nm. It should be noted that functionalized HCPT NPs had a light-color membrane surrounding the core and a slightly bigger diameter than the original HCPT NPs as evidenced by TEM image.

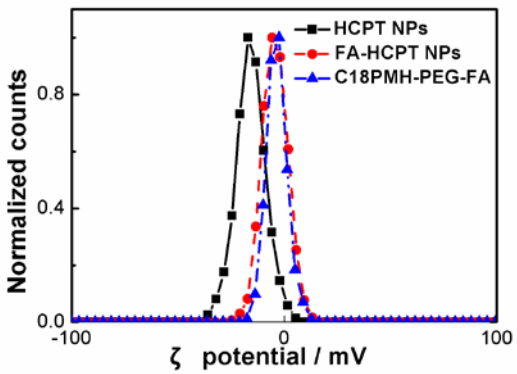

Fig. S3 Surface charge of HCPT NPs, functionalized HCPT NPs and C18PMH-PEG-FA (dispersed in water). We found that the mean ζ potential were -15.90 ± 0.50 mV, -4.10 ± 0.22 mV and -3.70 ± 0.50 mV for the HCPT NPs, FA-HCPT NPs and C18PMH-PEG-FA respectively.

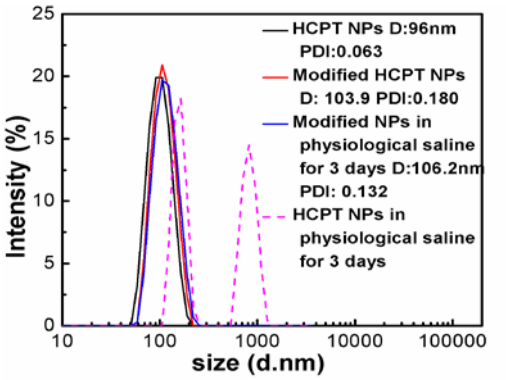

Fig. S4 Size distribution of the functionalized HCPT NPs by DLS measurements at 25 °C in water and stability of functionalized HCPT NPs in physiological saline.

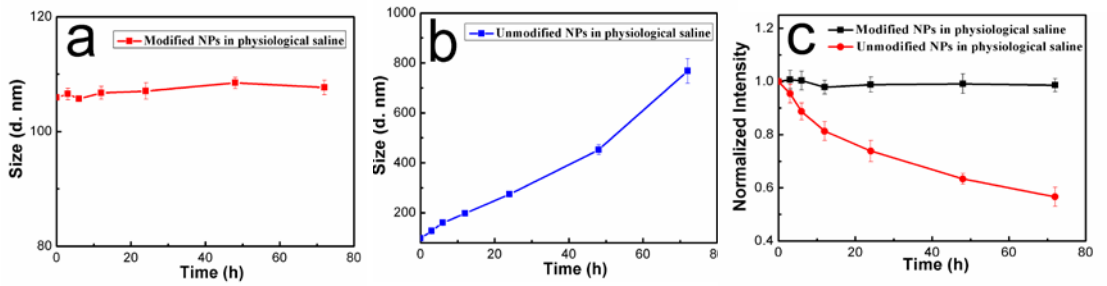

Fig. S5 (a) Stability studies of modified HCPT NPs in physiological saline. (b) Stability studies of the unmodified HCPT NPs in physiological saline. (c) Fluorescence comparison of the HCPT NPs and modified NPs in physiological saline.

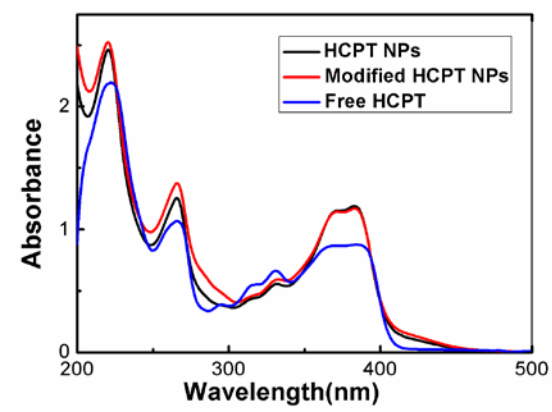

Fig. S6 UV-vis absorbance spectra of HCPT NPs, modifieded HCPT NPs suspensions and Free HCPT (dissolved in C_2H_5OH). UV-vis spectra show similar traces for both HCPT NPs and modifieded HCPT NPs indicative of their identical nature, implying that expectedly the HCPT NPs core remains unchanged by the attachment of the PEG.

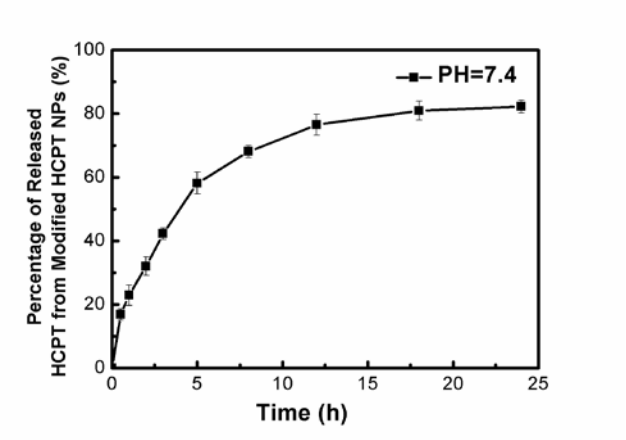

Fig. S7 Time-dependent release of HCPT from functionalized HCPT NPs released into a PBS solution (pH 7.4) at 37 °C for 24 h. Data were obtained by measuring thefluorescence intensity of free HCPT in solution (λem = 550 nm; λex = 382 nm). Error bars were based on standard deviations of triplicate samples.

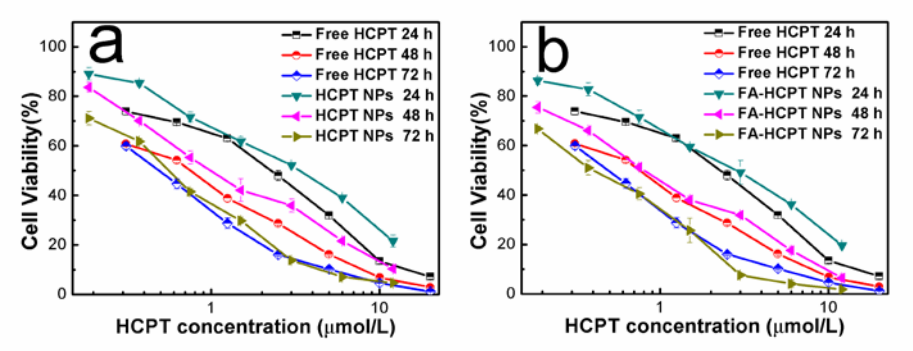

Fig. S8 *In vitro* cell toxicity assay. Concentration-dependent and time-dependent cell survival data of KB cells treated with free HCPT, HCPT NPs and FA-HCPT NPs. Viability of KB cells following incubation of free HCPT, HCPT NPs and FA-HCPT NPs and viability was measured using an MTT assay. Error bars were based on standard deviations of three parallel samples at each data point.

Table S1. Half-Maximal Inhibitory Concentration (IC50/μM) Values of Free HCPT, HCPT NPs, and FA-HCPT NPs KB Cell Lines.

	24 h	48 h	72 h
Free HCPT	2.50 ± 0.13	0.94 ± 0.06	0.49 ± 0.02
HCPT NPs	3.30 ± 0.18	1.85 ± 0.09	0.55 ± 0.03
FA-HCPT NPs	2.82 ± 0.15	1.53 ± 0.10	0.50 ± 0.03

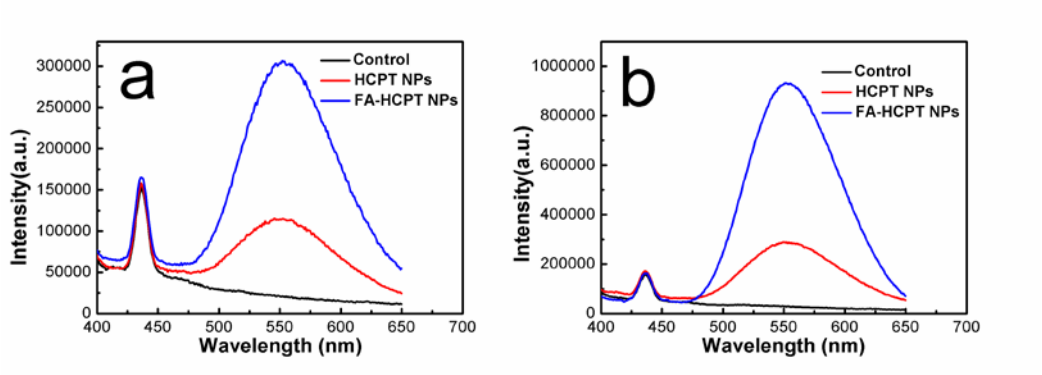

Fig. S9 Fluorescence measurements on cellular internalization of HCPT NPs and FA-HCPT NPs into FR(+) KB cells following 2 h (a) and 8 h (b) incubation(the concentration of HCPT was 6 μM in all samples) under 382 nm excitation. The plain FR(+) KB cells were used as control.

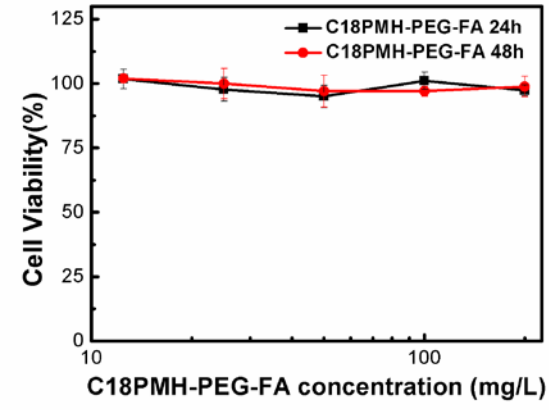

Fig. S10 Concentration-dependent cell survival data of KB cells treated with plain C18PMH-PEG-FA solution. Cells were incubated with a series of concentrations of C18PMH-PEG-FA for 24 and 48 h, and viability was measured using an MTT assay. Error bars were based on standard deviations of three parallel samples at each data point.