Transferrin Receptor-targeted Redox/pH-sensitive Podophyllotoxin Prodrug Micelles for Multidrug-Resistant Breast Cancer Therapy

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1 Materials

Mal-PEG-NH_2 and mPEG-NH_2 (molecule weight= 2,000 Da) was purchased from JENKEM Technology Co., Ltd. (Beijing, China). N-terminus cysteine modified T7 peptide (Cysteine-Histidine-Alanine-Isoleucine-Tyrosine-Proline-Arginine-Histidine, CHAIYPRH; named as Pep) was obtained from GL Biochem Peptide Ltd. (Shanghai, China). Podophyllotoxin (PPT), acetyl chloride, 3,3′-dithiodipropionic acid (DTPA), succinic anhydride (SA), 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC·HCl), and N-hydroxysuccinimide (NHS) were purchased from Aladdin Industrial Corporation (Shanghai, China). The docetaxel (DTX) and paclitaxel (PTX) were purchased from Energy Chemical (Shanghai, China). 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) were purchased from Beyotime Biotechnology (Shanghai, China).

2 Cells and animals

MCF-7 cells (Human breast cancer cell line) and A549 cells (Human non-small cell lung cancer cell line) were purchased from Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). MCF-7/ADR cells (Human breast cancer doxorubicin-resistant cell line) and A549/PTX cells (Human non-small cell lung cancer paclitaxel-resistant cell line) were obtained from KeyGEN BioTECH (Nanjing, China).

BALB/c nude mice (female, 4-6 weeks, 18 ± 2 g) and Kunming mice (female, 6-8 weeks) were purchased from the Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). All animals received care in compliance with the guidelines outlined in the Guide for the Care and Use of Laboratory Animals and all procedures were approved by The Jiangsu Province Hospital of TCM Care and Use Committee.

3 Characterization

^{1}H NMR spectra were carried on a Bruker AVANCE Ⅲ 300 MHz NMR spectrometer in DMSO-*d6* or D_2O. PPT and PPT-prodrug were determined by high-performance liquid chromatography (HPLC, Shimadzu, LC-20A, Japan) with the

detector set at 292 nm using acetonitrile and water (80: 20, v/v) as a mobile phase. The nanoparticle size, polydispersity index (PDI), and surface zeta potential were investigated using dynamic light scattering (DLS; Zs90; Malvern, UK). Critical micelle concentration (CMC) was measured according to the previous method using Nile red as the fluorescent probe.[1]

4. Serum stability and hemolysis assay

The stability of Pep-SS-NPs in serum was investigated by incubating micelle solution in PBS (pH 7.4) at 37 ℃ with or without 10% FBS. The average size of Pep-SS-NP micelle was tested at intervals using DLS.

The hemocompatibility of Pep-SS-NPs at different concentrations was assessed by hemolysis assay.[2] In brief, freshly mice blood was diluted by PBS (pH 7.4), and red blood cells (RBCs) were collected by centrifugation. After carefully washing and diluting, 2% RBC suspension was used for hemolysis study. Then, PPT prodrug micelle solution at systematically varied concentrations were added and mixed by vortex and incubated at 37 °C for 2 h. After that, the mixtures were centrifuged at 1200 rpm for 10 min. The supernatant was collected and the amount of hemoglobin released was recorded on a microplate plate reader (Bio-Rad, CA, USA) at 540 nm.

Double-distilled water was used as positive control and PBS was used as negative control. The hemolysis ratio (HR) of RBCs was calculated according to the following formula:

Hemolysis (%) = $(A_{sample} - A_{negative}) / (A_{positive} - A_{negative}) \times 100\%$, where, A_{sample} is the absorbance of sample, $A_{negative}$ is the absorbance of negative control and $A_{positive}$ is the absorbance of positive control. All hemolysis experiments were carried out in triplicate.

5. Maximum tolerated dose

Kunming mice were divided into 10 groups (n = 10) and administered intravenously with the free PPT (5, 10, 15, 20, 30 mg/kg) or Pep-SS-NPs (20, 40, 80, 120, and 160 mg/kg in PPT equivalent). Changes in body weight and survival of mice were measured every other day for 2 weeks. The maximum tolerated dose (MTD) was identified as the maximum dose of a drug that dose not induce animal death or >20%

body weight loss or other remarkable changes in the general appearance within the entire period of the experiments.

Supplementary Figures

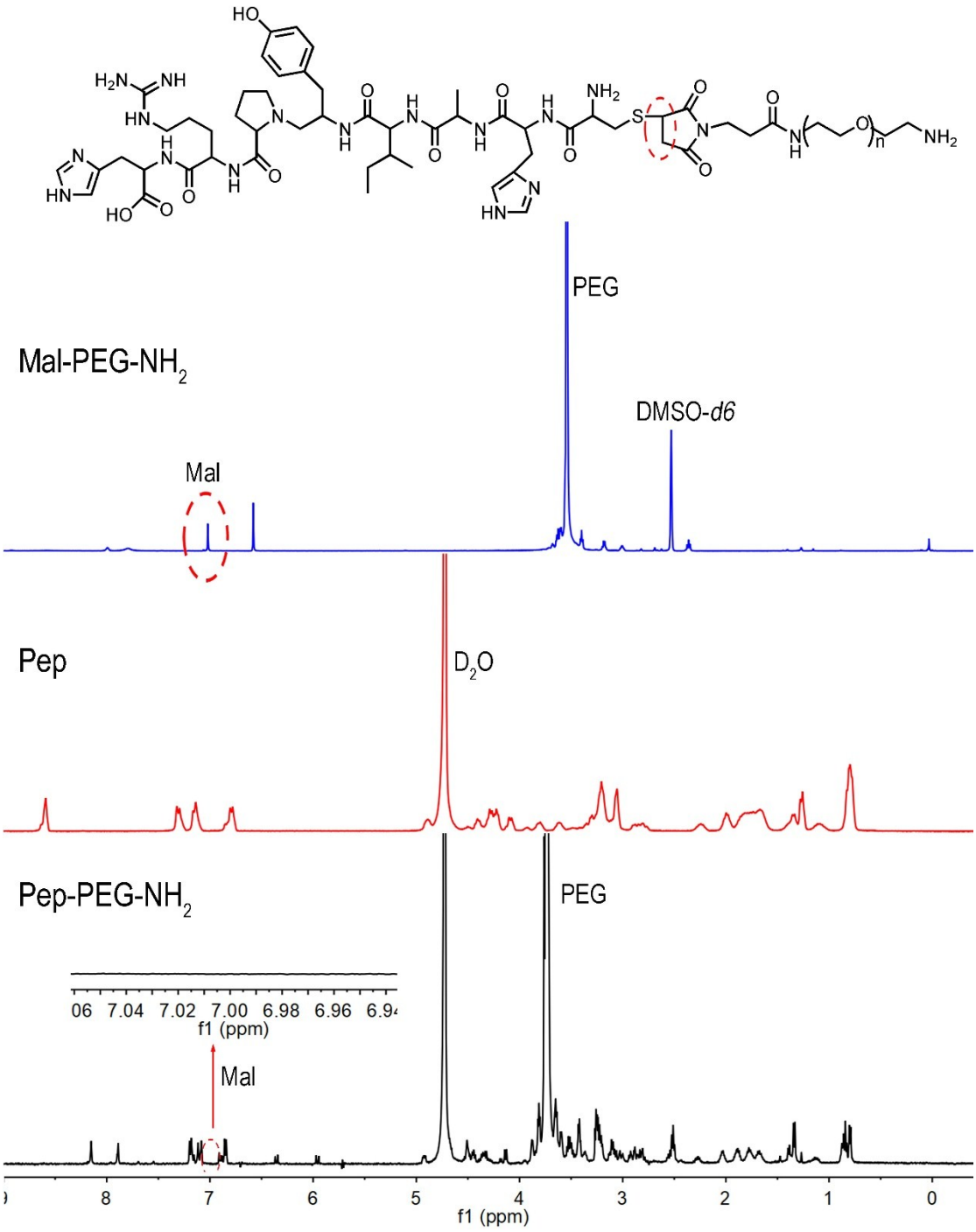
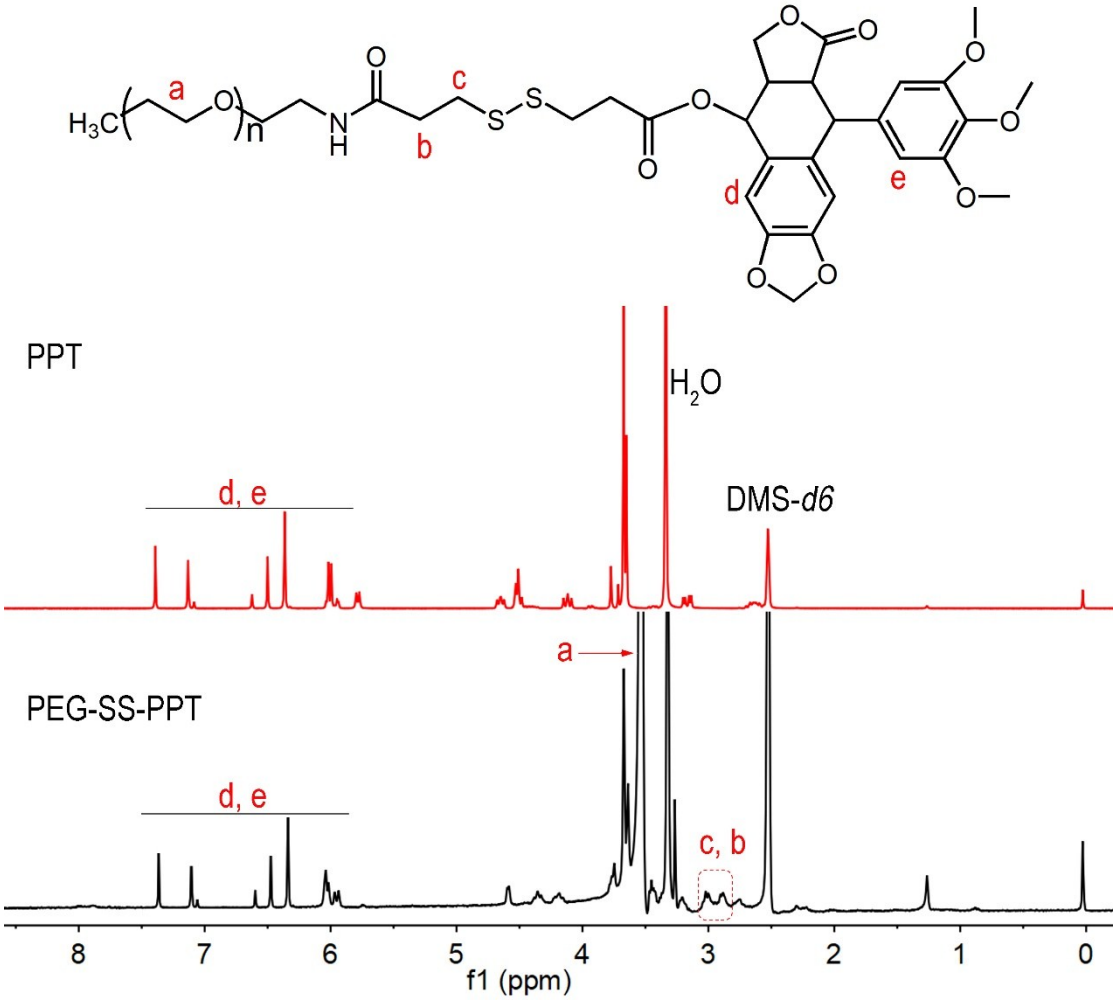


Fig. S1 ^{1}H NMR spectrum of Mal-PEG-NH_2 and Pep-PEG-NH_2 in D_2O.



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94 **Fig. S2** ^{1}H NMR spectrum of PPT and PEG-SS-PPT in DMSO-*d6*.

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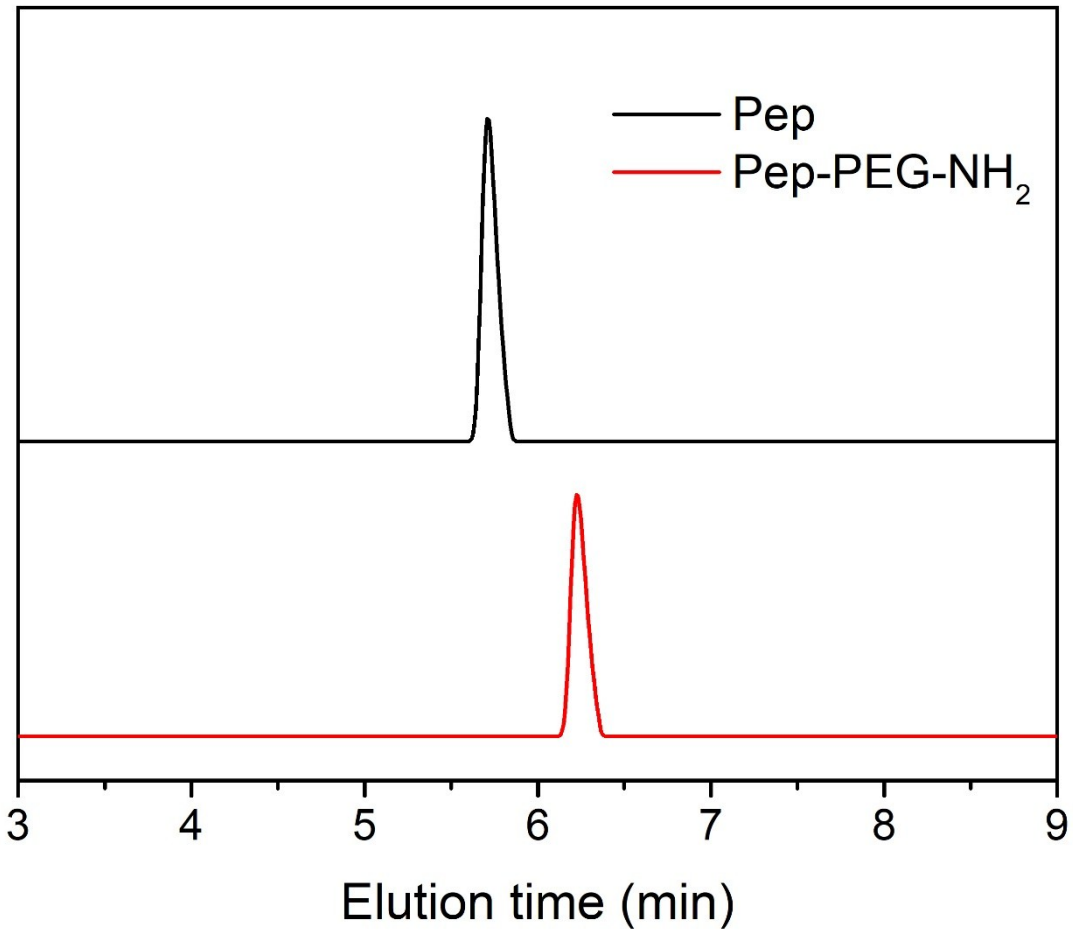

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97 **Fig. S3** HPLC spectrum of Pep and Pep-PEG-NH_2.

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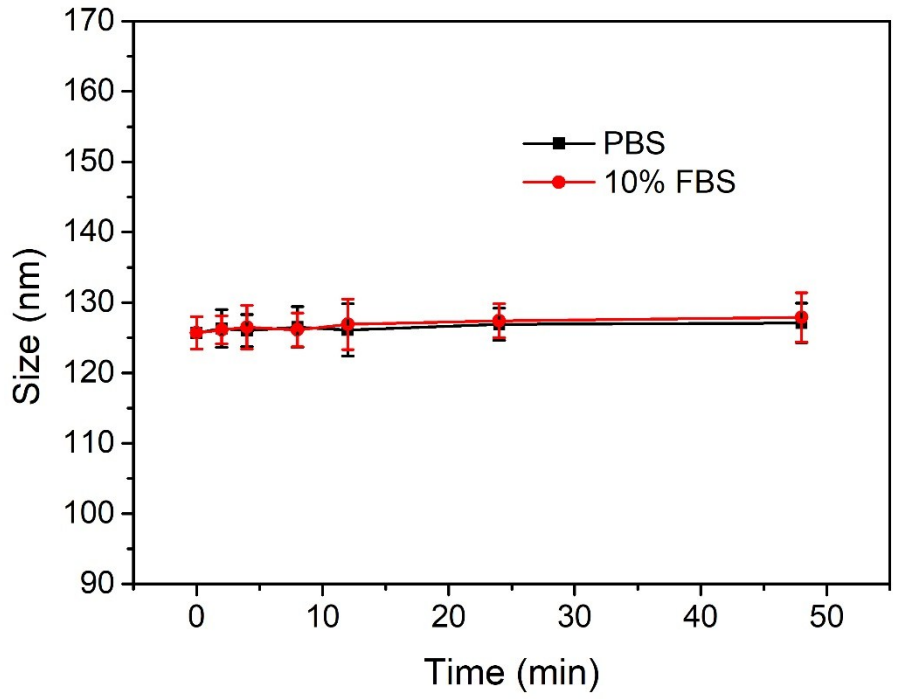


Fig. S4 Size changes of Pep-SS-NPs in pH 7.4 with or without 10% FBS, data shown as mean ± SD, $n = 3$.

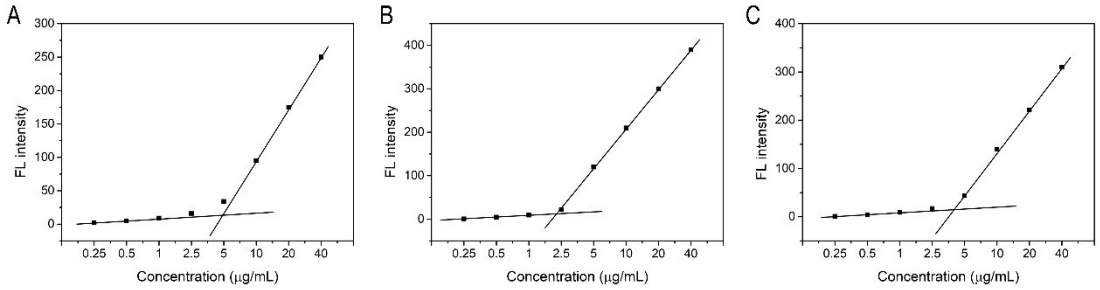
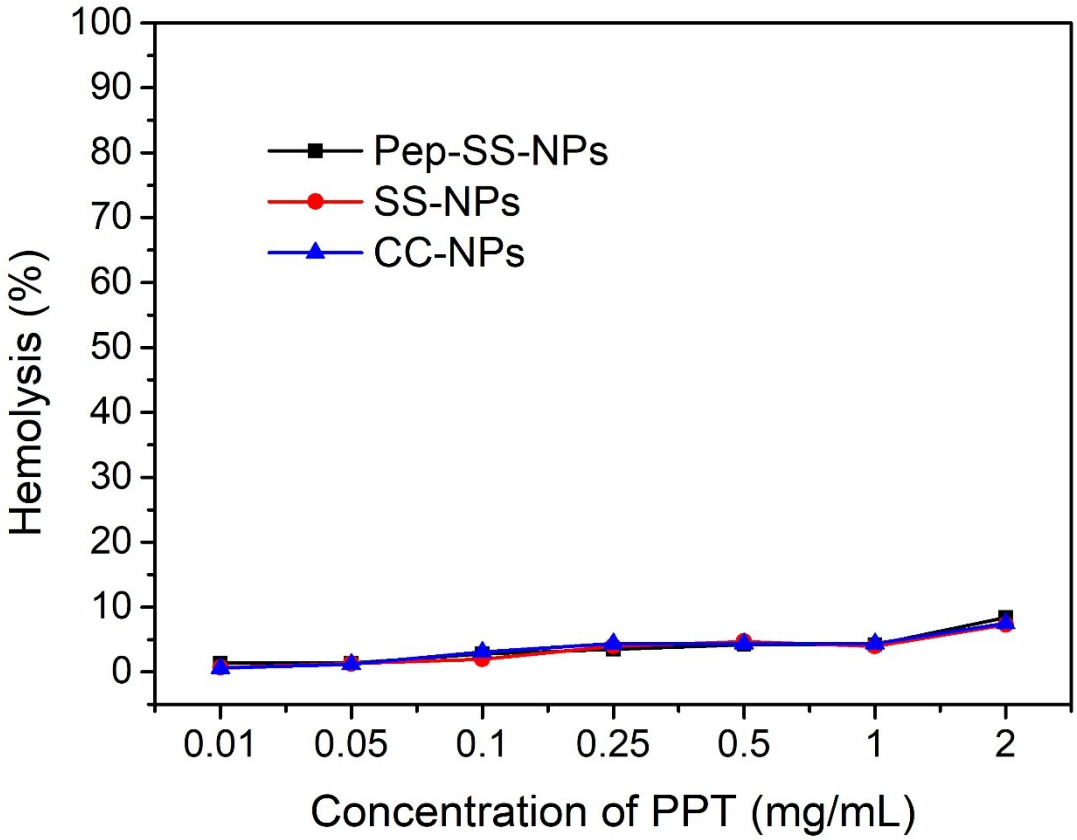


Fig. S5 CMC of Pep-SS-NPs (A), SS-NPs (B), and CC-NPs (C).



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107 **Fig. S6** Hemolysis rates of Pep-SS-NPs, SS-NPs, and CC-NPs at the range of 0.1 to 2.0 mg/mL of

108 PPT. Data presented as mean ± SD, $n = 3$.

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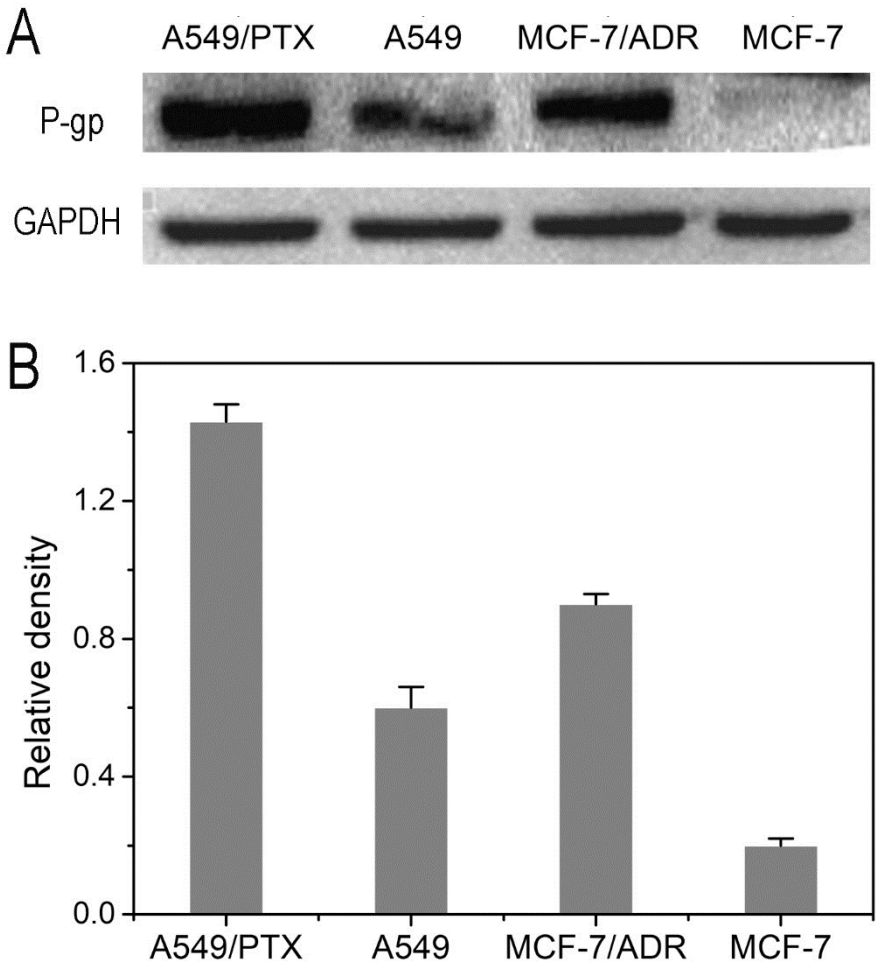

Fig. S7 Western P-gp expression in A549/PTX, A549, MCF-7/ADR, and MCF-7 cells determined by western blotting using GAPDH as an internal control.

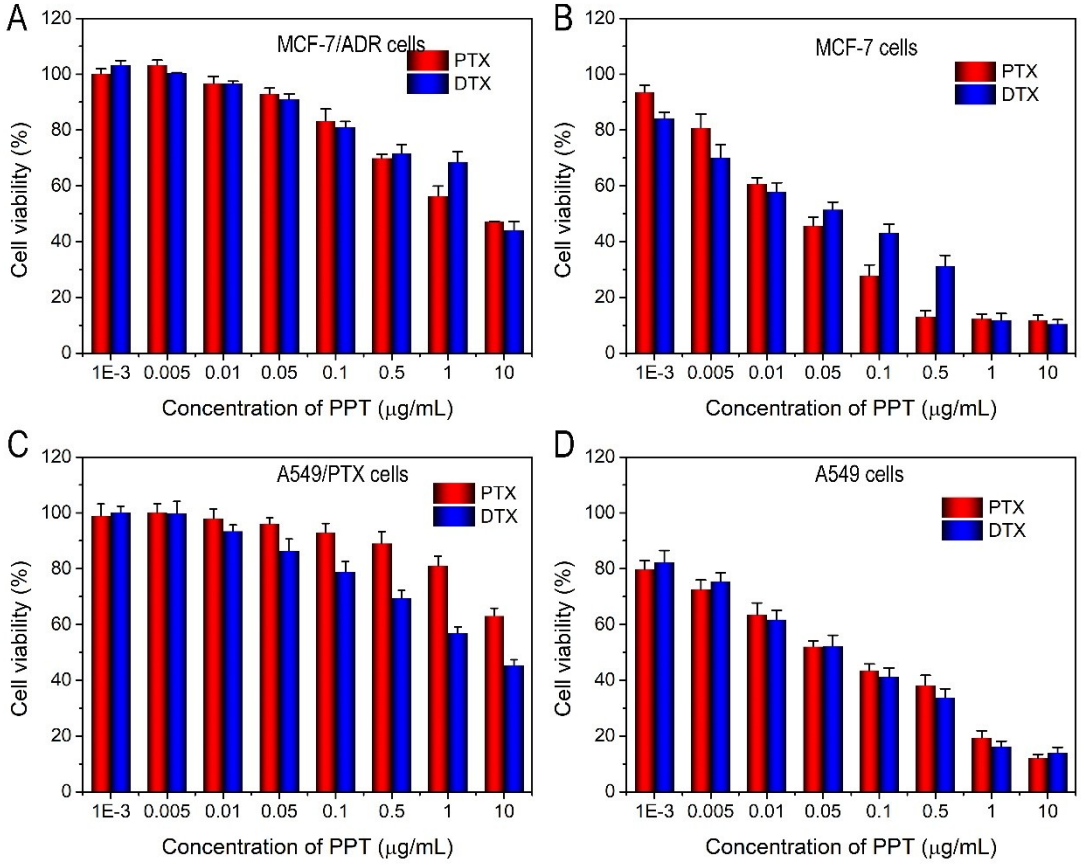

Fig. S8 Cytotoxicity analysis of PTX and DTX on MCF-7/ADR cells (A), MCF-7 cells (B), A549/PTX cells (C), and A549 cells (D) for 48 h by MTT assay. Data shown as mean ± SD, $n = 6$.

References

1. K. Cai, X. He, Z. Song, Q. Yin, Y. Zhang, F. M. Uckun, C. Jiang and J. Cheng, *Journal of the American Chemical Society*, 2015, **137**, 3458-3461.
2. J. Zhu, Q. Huo, M. Xu, F. Yang, Y. Li, H. Shi, Y. Niu and Y. Liu, *Nanoscale*, 2018, **10**, 10.1039.C1038NR03899F-.

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