Supplementary Information For

Docetaxel-loaded PEO–PPO–PCL/TPGS mixed micelles for overcoming multidrug resistance and enhancing antitumor efficacy

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Cell culture

MCF-7/Adr, MCF-7 and A549 cells were cultivated in 75 cm^3 flasks a humidifid 5% CO_2/95% atmosphere incubator at 37 °C. Cells were cultured in RPMI medium, supplemented with 10% fetal bovine medium (FBS). The medium was changed every other day. The cells were subcultured 2-3 times per week with 0.25% trypsin-EDTA. Before experiment, the cells were cultured until confluence was reached to about 75%.

The cellular uptake and flow cytometric (FCM) analysis

The cells were cultured as described above for cellular uptake studies. A549 and MCF-7 cells were seeded on six-well culture plates (1×10^5 cells/well) and incubated 24 h in RPMI 1640 medium containing 10% FBS. The cells were incubated in the medium at 37 **°C** with different treatments (with an equivalent C6 concentration of 5 μg/mL). After an incubation period of 2 or 4 h, extracellular C6 was removed by PBS. The cells were imaged using inverted fluorescence microscope (Olympus, Tokyo, Japan). Flow cytometry was utilized to quantify the cellular uptake. The cells were harvested by trypsinization with centrifugation (1000 rpm, 5 min). Finally, the cells were suspended in 500 μL of PBS and stored on ice until analysis. The fluorescence

intensity in the cells was determined using a flow cytometry (Becton Dickinson, San Jose, CA). The individual fluorescence of 10^4 cells was collected for each sample.

MTT assay

In brief, MCF-7 and A549 cells were cultured in 96-well plates with a density about 5000 cells/well. Cells were adherent 24 h and cultured with fresh media containing treatment drugs including free DTX solution, DTX-loaded MM. The concentrations of DTX in the assay were ranging from 0.025 μg mL^{-1} to 25 μg mL^{-1} (0.025, 0.25, 2.5, 12.5, 25 μg mL^{-1}). The samples were added to cells for 24, 48 and 72 h of co-culturing. Each sample was performed in sextuplicate. Then 20 μL of 5 mg mL^{-1} MTT dissolved in PBS was added to each well. The plates were incubated for another 4 h at 37 °C and then the medium was discarded. Then, the formazan crystals were dissolved in 150 μL of DMSO. The absorbance of the wells was tested by the microplate reader (Bio-Rad680, USA) at 570 nm wavelength. Meanwhile, the cytotoxicity of the blank micelles were also tested with the same method. The concentrations were ranging from 0.5 μg mL^{-1} to 500 μg mL^{-1} (0.5, 5, 10, 250, 500 μg mL^{-1}).

Propidium iodide (PI) staining process

MCF-7 and A549 cells were seeded on six-well culture plates (1×10^5 cells/well) and incubated overnight in RPMI (Roswell Park Memorial Institute) 1640 containing 10% FBS. Free DTX and DTX-loaded MM (2.5 μg mL^{-1}) were put into the wells, respectively. After 24, 48 and 72 h incubation, the morphology of the cells were observed using inverted fluorescence microscope (Olympus I×71). For PI staining, the control (untreated) and treated cells (mentioned above) were harvested and washed twice with cold PBS, subsequently, cells were collected by centrifugation and gently resuspended in binding buffer (containing hepes, NaCl and $CaCl_2$), and stained with PI (100 μg mL^{-1}, 0.5 mL) in the dark for 10 min at 37 °C. Then cells were washed twice to remove the unbound PI. Finally, 2 mL PBS was added into the

samples before they were observed under an inverted fluorescence microscope (I×71, Olympus, Tokyo, Japan).

Table S1 The scattering intensity of DTX-loaded MM stored at 4 °C for 3 months (mean ± SD, n=3).

Formulations	Intensity (cps)						
Time/month	0	0.5	1.0	1.5	2.0	2.5	3.0
DTX-loaded P9/TPGS	12148±230	10894±118	10925±118	10627±220	9951±116	10013±120	11069±185
DTX-loaded P18/TPGS	11148±123	12394±108	11925±200	10907±17	10951±230	10069±239	11309±109
DTX-loaded P36/TPGS	10156±181	10494±148	10525±136	9827±223	9998±236	10737±146	11169±99

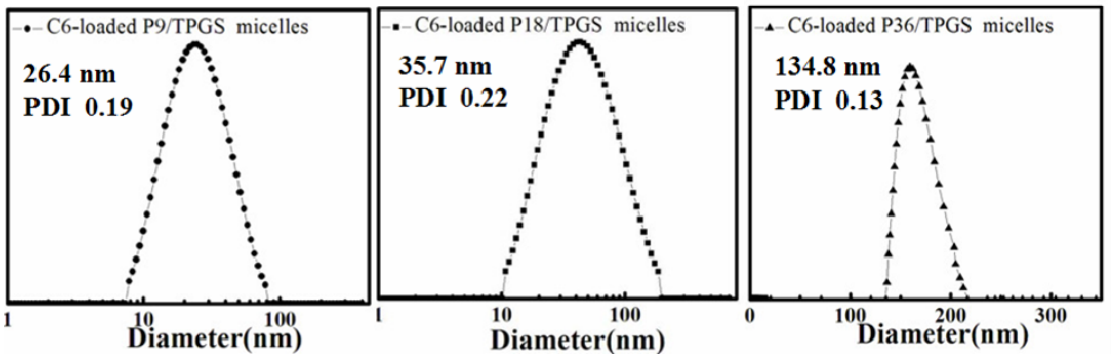

Fig. S1. Size and size distribution of the C6-loaded PEO-PPO-PCL/TPGS micelles

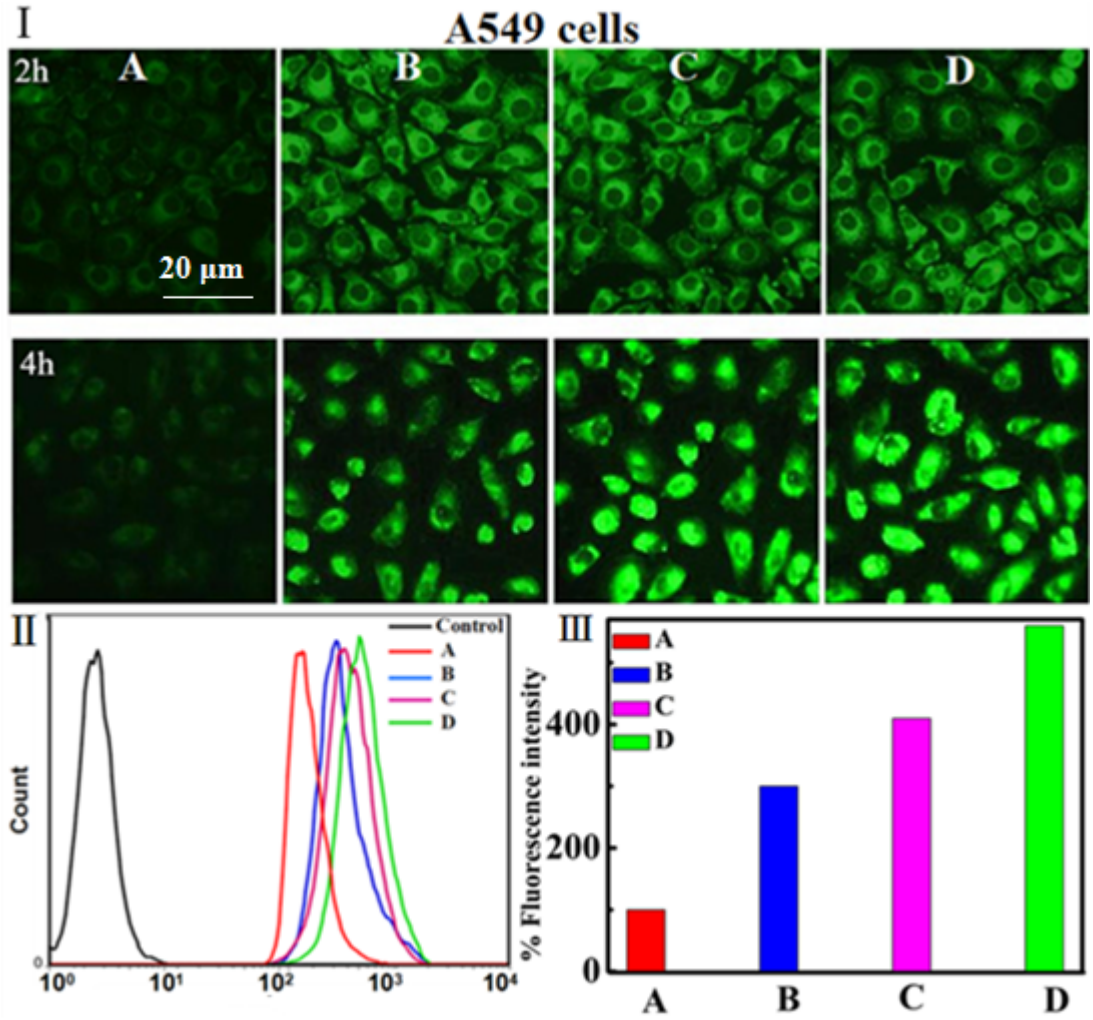

Fig. S2. Fluorescent micrographs (I, for 2h and 4h), flow cytometric profiles of intensity (II) and corresponding quantitative histogram profiles (III) of A549 cells incubated for 4 h in different samples. (A) free C6, (B) C6-loaded P9/TPGS MM, (C) C6-loaded P18/TPGS MM, (D) C6-loaded P36/TPGS MM. The fluorescence intensity of free C6 was expressed as 100%.

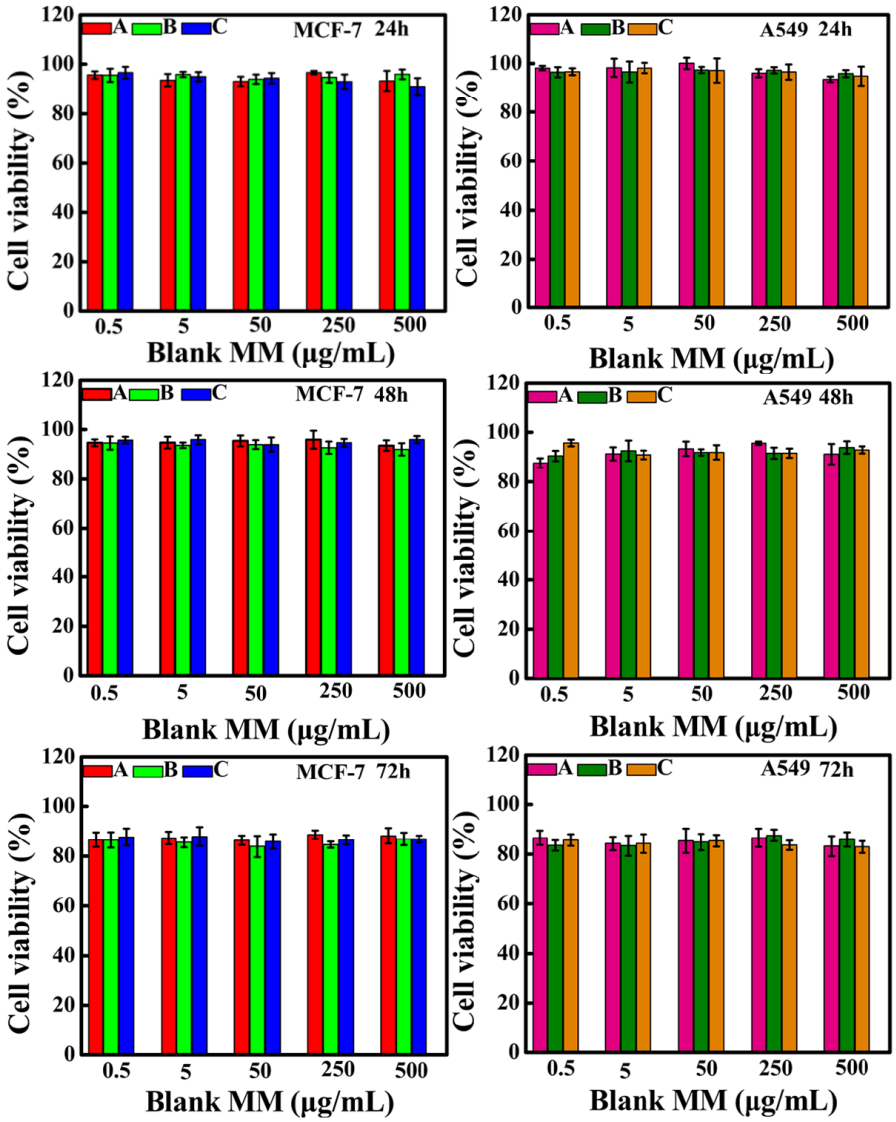

Fig. S3. The MCF-7 cells and A549 cells viability after incubation with blank MM for 24 h, 48 h and 72 h. (A) P9/TPS MM, (B) P18/TPGS MM, (C) P36/TPGS MM (mean ± SD, n=6).

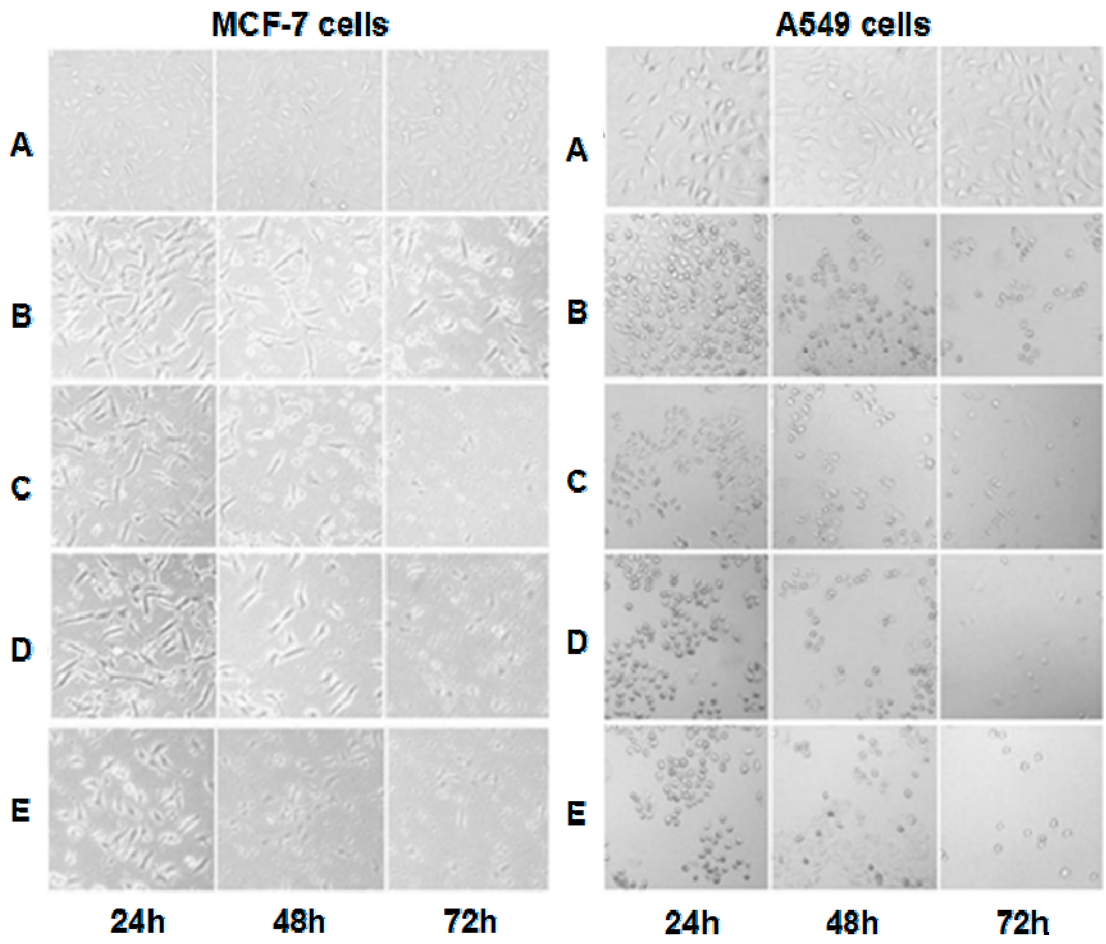

Fig. S4. Drug induced morphology changes. (A) control cells, (B) free DTX, (C) DTX-loaded P9/TPGS MM, (D) DTX-loaded P18/TPGS MM, (E) DTX-loaded P36/TPGS MM.

Supplementary materials and methods

Hoechst 33342 were purchased from Sigmae-Aldrich (China).

Apoptosis analysis (hoechst 33342 Staining)

Hoechst 33342 has been shown to be a useful marker for cellular apoptosis. Nuclear morphology of MCF-7 and A549 cells with different treatments was observed by hoechst 33342 staining method. MCF-7 and A549 cells were seeded on a 12-well culture plate (100,000 cells/well) and incubated for 12 h in 2 mL of RPMI 1640 medium containing 10% FBS in a humidified atmosphere with 5% CO_2 at 37 °C, then cells were incubated with medium containing free drug solution or micelles at the

total DTX concentration of 10 μg mL^{-1} for 24 h. Samples were then stained with hoechst 33342 solution (10 μg mL^{-1}) in the dark for 15 min at 37 °C, and then were triple washed with cold PBS. The cells were finally observed by an inverted fluorescence microscope (I×71, Olympus, Tokyo, Japan)

Supplementary result

Cell apoptosis is also an important index for evaluating the *in vitro* anti-tumor effect. In order to elucidate DTX formulations induced-apoptosis in MCF-7 and A549 cells hoechst staining of nuclei is observed after different treatments.[1] As shown in Figure. S3, the nuclei of control cells are homogenous fluorescence and show a good integrity with no evidence of segmentation and fragmentation after hoechst 33342 staining (Figure. S5 A), while the cells treated with free DTX or DTX-loaded micelles (Figure. S5 B-E) exhibit chromatin condensation and nuclear fragmentation, indicating breakdown in the chromatin followed by DNA condensation. These results also indicate that, the DTX-loaded MM have an enhanced apoptosis effect in both cell lines than free DTX.

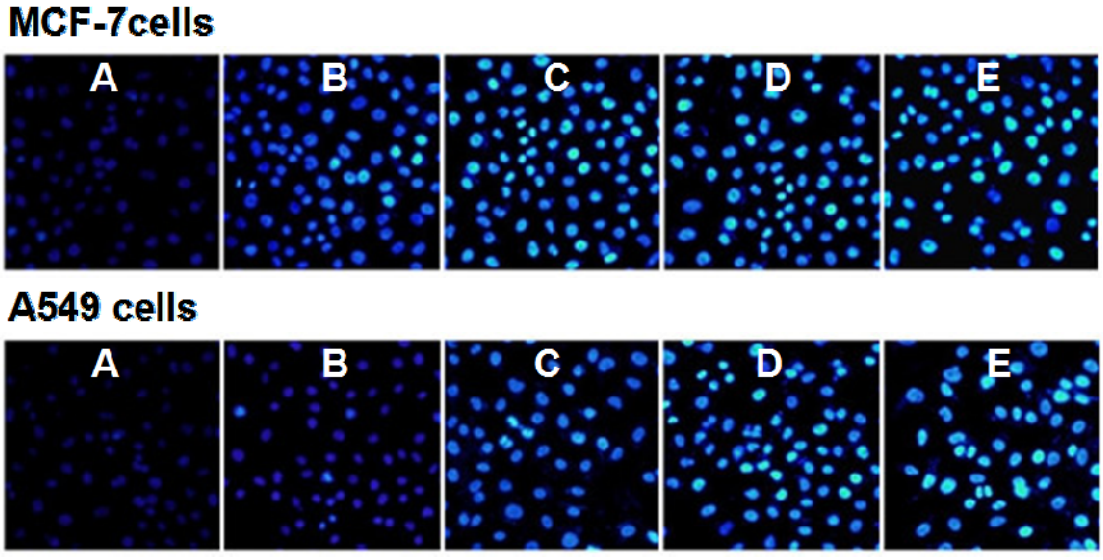

Fig. S5. Nuclear morphologies of MCF-7 and A549 cells using hoechst 33342 staining. (A) control cells, (B) free DTX, (C) DTX-loaded P9/TPGS MM, (D) P18/TPGS MM, (E) P36/TPGS MM.

Supplementary references

[1] F. Wang, D. Zhang, Q. Zhang, Y. Chen, D. Zheng, L. Hao and Y. Liu, *Biomaterials*, 2011, **32**, 9444-9456.