Supplementary material

The influence of nanocarrier architectures on antitumor efficacy of docetaxel nanoparticles

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Materials and Methods

Materials

Copolymer $G2C_{18}$ (MW = 2674) and $PEG_{45}C_{18}$ (MW = 2254) were synthesized according to previous paper.[57] Docetaxel (DTX, purity > 98%) was purchased from Beijing Coupling Biotechnology Co., Ltd., DTX injection was obtained from Xiyuan Hospital, N, N-dimethylformamide (DMF) was purchased from Beijing Chemical Plant. Sodium lauryl sulfate (SDS) was purchased from National Group Chemical Reagent Co., Ltd., RPMI 1640 medium, PBS buffer, 0.25% trypsin were purchased from HyClone Company of the United States. Fetal bovine serum (FBS), tetracycline double antibody were purchased from Gibco Thermo Scientific (United States), 0.9% sodium chloride injection (batch number: H17092210) was purchased from Shandong Hualu Pharmaceutical Co., Ltd. Paclitaxel injection was purchased from Peking Union Pharmaceutical Co., Ltd. Acetonitrile and methanol were chromatographically pure, and other reagents were analytically pure.

Animals and cell line

The murine breast cancer cell line (4T1 cell line) was purchased from National Infrastructure of Cell Line Resource (Beijing, China) and incubated in RPMI-1640 medium, 10% fetal bovine serum, 100 units mL^{-1} of penicillin G, and streptomycin with 5% CO_2 atmosphere at 37 °C. BALB/c mice (20 ± 2 g) were obtained from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) and raised in a laminar flow room at 25 ± 2 °C for 1 week prior to experimentation. All experimental procedures were performed according to the Guidelines and Policies for Ethical and Regulatory for Animal Experiments and approved by the Animal Ethics Committee of Peking Union Medical College (Beijing, China).

Preparation of DTX-loaded nanoparticles

DTX (16 mg) and nanocarriers ($G2C_{18}$ or $PEG_{45}C_{18}$, 4 mg) were dissolved in DMF (0.5 mL) with the ratio of drug vs. stabilizer of 4: 1, and then added into deionized water (8 mL) dropwise under the condition of continuous ultrasonic treatment, the mixture was dialyzed using dialysis membrane (MWCO 8000-14000) against deionized water (1 L h^{-1} × 4 h) to remove the organic solvent and free drug. Then, the obtained nanoparticle solutions were homogenized at 25 °C for 5 times under 1600 bar pressure. Ultimately, the quantitative analysis of DTX in these nanoparticles (DTX/$G2C_{18}$ nanoparticles and DTX/$PEG_{45}C_{18}$ nanoparticles) was detected by HPLC (UltiMate3000, Thermo) with a Waters Symmetry C_{18} column (250 mm × 4.60 mm, 5 μm) using a UV detector at 230 nm, the calibration curve was generated from acetonitrile/water (acetic acid, 0.1%) (65/35, v/v) ($y = 0.6828x + 0.1958$, $R^2 = 0.9999$). The injection volume was 20 μL, and the flow rate was 0.8 mL min^{-1}. The drug-loading content (DLC) was calculated according to the equation as follows:

$$DLC(\%) = \frac{\text{weight of loaded drug}}{\text{weight of drug - loaded nanoparticles}} \times 100\%$$

Particle size and zeta potential measurement

The mean diameter, particle size distribution, and zeta potential of DTX/$G2C_{18}$ nanoparticles and DTX/$PEG_{45}C_{18}$ nanoparticles were detected by Zeta Sizer Nano ZS analyzer (Malvern Instruments, UK) at 25 °C, which integrated Phase analysis Light Scattering ($\lambda = 633$nm) and the Non-Invasive Backscatter optics (scattering angle $\theta = 173°$).

Scanning electron microscopy

The morphology of DTX nanoparticles were investigated using scanning electron microscopy (SEM, S-4800, Hitachi Limited, Japan). A drop of DTX nanoparticle solution (100 μg mL^{-1}) was placed onto matrix and air dried. After sputter-coating with Au/Pd for 1 min, samples were observed at 30 mV accelerating potential.

Storage stability

In order to study the storage stability, DTX nanoparticles were stored at 4 °C for 4 weeks. The particle size was measured at 0, 1, 3, 5, and 7 days, respectively. The experiment was carried out in triplicate.

Stability in media

The stability of DTX nanoparticles in different media was investigated at 37 °C, including normal saline solution, 5% glucose solution, PBS solution (pH 7.4), and plasma. The particle size of the sample was measured at 0, 2, 4, and 6 h. The experiment was carried out in triplicate.

Drug release profile

DTX nanoparticles, and DTX solution of 1 mg mL^{-1} (DTX equivalent concentration) were placed into the dialysis bag (MWCO = 8000-14000), and then immersed in 50 mL PBS solution (pH 7.4) containing

0.5% SDS under the condition of 37 °C and continuous magnetic stirring. At predetermined time intervals of 0.25, 0.5, 1, 2, 4, 8, 12, 24, 48, 72, 96, 120, 144, 168, 192 h, the outside release medium (1 mL) was taken out for analysis, meanwhile the fresh release medium with the same volume was supplemented, respectively. Replace all released medium every 24 h. Drug release studies were conducted for 8 d, and all release experiments were carried out in triplicate. The concentration of DTX in release medium was quantified by HPLC, and the cumulative release rate of DTX was calculated and the release curve was drawn.

Investigation on erythrocyte membrane toxicity

The blood was taken from the eyeball of BALB/c mice. After removing the plasma by 5000 rpm centrifugation for 5 min, the erythrocytes were collected and suspended in saline solution to prepare 4% erythrocyte (RBC) solution. DTX nanoparticle solution with the concentration ranging from 0.06 to 1.00 mg mL^{-1} (DTX equivalent concentration) was mixed with 4% RBC suspension in equal volume, and then incubated at 37 ^{o}C for 4 h (tested group). The samples were centrifuged to remove the RBCs, then 150 μL of the supernatant was transferred to the 96-well plate, and the absorbance (A) value at 540 nm was measured using an ELISA plate reader. Assuming that 100% hemolysis was caused by deionized water (positive control) and 0% hemolysis caused by saline solution (negative control), the hemolysis rate was calculated.

MTT assays

The cytotoxicity of DTX nanoparticles on murine breast cancer cell line (4T1 cell line) *in vitro* was determined by a MTT method. Under the condition of 37 °C and 5% CO_2, 4T1 cells were inoculated in 96-well plate at a density of 1×10^4 cells per well in RPMI-1640 medium supplemented with 10% fetal bovine serum and 0.1% double antibody. After incubating for 24 hours, the growth medium was changed with fresh medium, and then, DTX injection, DTX/G2C_{18} nanoparticles, and DTX/$PEG_{45}C_{18}$ nanoparticles with different concentration (diluted using RPMI-1640 medium) were added into the each well (150 μL per well). After incubation 48 h, 20 μL MTT solution (5 mg mL^{-1}) was added to each well and incubated for another 4 h. After removing the culture medium and adding 200 μL DMSO solution per well, the formazan was dissolved by fully vibrate for 10 min. The absorbance (A) value of the solution in each well was measured at 570 nm using an ELISA plate reader.

Cell inhibition rate (%) = (1-tested group A value/ control group A mean value) × 100%, in which tested group A value was obtained from cells treated with different concentration of DTX samples, control group A value was obtained from cells cultured with RPMI-1640 medium. The half inhibitory concentration (IC_{50}) was calculated according to the results of MTT.

Cellular uptake

4T1 cells (5×10^5 cells per well) were inoculated in 12-well plate and cultured in RPMI-1640 medium at 37 °C for 48 h under the condition of 5% CO_2, and then, 1 mL solution of sucrose, hydroxypropyl-CD, and cytochalasin D in RPMI-1640 medium as endocytosis inhibitors were added separately, and

then incubated for another 1 h. DTX injection, DTX nanoparticles solutions (DTX equivalent concentration 50 μg mL^{-1}, 1 mL) were added into the 12-well plate. After incubation 3 h, the culture medium was removed and the cells were washed with PBS for three times. Then the cells were digested with cell trypsin and the cell precipitates were collected again. After adding 0.2 mL RIPA lytic buffer and 1 mL ethyl acetate, the mixture was centrifuged at 10000 rpm for 15 minutes. The supernatant was collected, evaporated, and dissolved in 200 μL methanol. The mixture was filtrated via a 0.22 μm filter, and the concentration of DTX in filtrate was detected by HPLC.

***In vivo* antitumor effect**

Mice carrying 4T1 tumor was selected as the model to evaluate the *in vivo* antitumor activity of DTX/G2C_{18} nanoparticles, and DTX/$PEG_{45}C_{18}$ nanoparticles. In short, 4T1 tumor was produced by subcutaneous injection of 0.2 mL 4T1 cell suspension (1×10^7 cells) into the right armpit of BALB/c mice (20 ± 2 g). When the tumor volume was over 100 mm^3, these mice were randomly divided into 4 groups (n = 8), which was treated with 0.2 mL normal saline solution (blank control group), DTX injection with a concentration of 10 mg Kg^{-1} (positive control group), DTX nanoparticles with a concentration of 10 mg Kg^{-1} (test group) via the tail vein administration every 2 d for 6 times. During the entire procedure, the body weight of mice was monitored and the tumor volume was measured. After scarifying, tumors were collected and weighted. The tumor inhibition rate was calculated:

IR% = (1 – tumor weight of tested group or positive control group/tumor weight of blank control group) × 100%

Statistical analysis

Data were presented as the mean values ± standard deviation (>3 independent experiments). One-way analysis of variance (ANOVA) (SPSS 22.0, USA) was used for statistical evaluation, and $p < 0.05$ was considered as significant.

Table S1. Results of DTX nanoparticles.

Sample	Size (nm)	PDI	Z (mV)	DLC (%)
DTX/G2C_{18}	255.6 ± 15.6	0.12 ± 0.04	29.1 ± 0.28	72.2 ± 0.6
DTX/$PEG_{45}C_{18}$	396.8 ± 16.1	0.14 ± 0.02	21.4 ± 0.07	67.7 ± 1.9

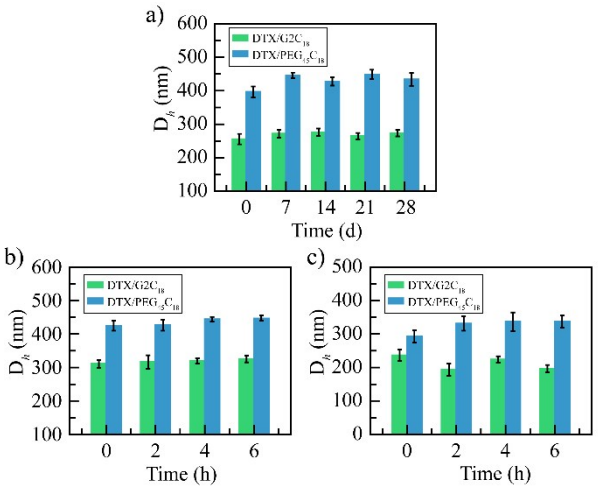

Fig. S1. Storage stability study of DTX nanoparticles at 4 °C (a), media stability study in 5% glucose solution at 37 °C (b), and plasma at 37 °C (c) (n = 3).

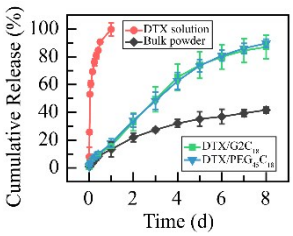

Fig. S2. The cumulative release curves of DTX nanoparticles (n = 3).