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Enhanced antitumor efficacy and decreased toxicity by self-associated docetaxel in phospholipid-based micelles

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ABSTRACT

To overcome the poor aqueous solubility of docetaxel (DTX) and the side effects of the emulsifier in the marketed formulation, we have developed a DTX-loaded micelle using a nontoxic and biodegradable amphiphilic diblock copolymer, methoxy polyethylene glycol-distearoylphosphatidylethanolamine ($mPEG_{2000}$-DSPE). The prepared micelles exhibited a core–shell structure, and DTX was successfully encapsulated in the core with an encapsulation efficiency of $97.31 \pm 2.95\%$ and a drug loading efficiency of $3.14 \pm 0.13\%$. The micelles were spherical with a hydrodynamic diameter of approximately 20 nm, which could meet the requirement for in vivo administration, and were expected to enhance the drug's antitumor efficacy and to decrease its toxicity. To evaluate the DTX-loaded micelles, we chose a well marketed formulation, Taxotere®, as the control. The prepared DTX micelle had a similar antiproliferative effect to Taxotere® in vitro but a significantly better antitumor efficacy than Taxotere® in vivo, which may be caused by passive targeting of the tumor by the micelles. In addition, the safety evaluation revealed that the DTX micelle was a qualified drug for use in vivo. Based on the experimental results, we propose that $mPEG_{2000}$-DSPE micelle is a potent carrier for DTX.

1. Introduction

In cancer chemotherapy, the use of anticancer drugs has been limited by their toxic side effects in normal organs and an inadequate concentration of the drug at tumor sites. Poor solubility in water is also an obstacle for the optimal clinical use of many anticancer drugs (Liu et al., 2008).

Docetaxel (DTX) is an important anticancer taxane that is currently used to treat malignant breast, ovarian, and lung tumors (Jakate et al., 2003). DTX has a similar structure to paclitaxel, and they both bind to the same site of tubulin. The affinity of DTX is 1.9-fold higher than that of paclitaxel (Bissery, 1995; Izquierdo et al., 2006). Because of its poor aqueous solubility, DTX is currently formulated as the marketed product Taxotere®, which contains ethanol and the nonionic surfactant Tween-80. However, Tween-80 has been shown to induce hypotension, tachycardia, and a rise in histamine levels in dogs (Eschalier et al., 1988) and has promoted the generation of biologically active complement products (Weiszhár et al., 2012). Clinically, the Tween-80 formulation is known to cause severe allergic reactions and peripheral neuropathy (Elsabahy et al., 2007; Liu et al., 2008). Therefore, the development of a new carrier that can solubilize DTX and avoid the side effects of Tween-80 is a current goal of antitumor drug research.

Polymeric micelles are composed of amphiphilic macromolecules that have distinct hydrophobic and hydrophilic block domains. In the hydrophilic segment, the most commonly used polymer is polyethylene glycol (PEG) that possesses a molecular weight of 2–15 kDa. In the hydrophobic segments, the most common materials are hydrophobic polyesters, but other materials, such as polyethers, polypeptides, poly(β-amino ester) or lipids, have also been utilized (Sutton et al., 2007; Kwon and Okano, 1996). In recent years, polymeric micelles have increasingly become the subject of scientific research. They have emerged as potential carriers for poorly water-soluble drugs because they can solubilize those drugs in their inner core, and they possess attractive characteristics, such as a generally small size (<100 nm) and a propensity to evade scavenging by the reticuloendothelial system (RES) (Jones and Leroux, 1999). In addition, passively targeting polymeric micelles to the solid tumor can be achieved through the enhanced permeability and retention (EPR) effect (Kedar et al., 2010; Gaucher et al., 2005).

In this work, we have developed a DTX micelle using a nontoxic and biodegradable amphiphilic diblock copolymer, $mPEG_{2000}$-DSPE (Cesur et al., 2009), to overcome the poor aqueous solubility of DTX and the side effects of Taxotere®. To evaluate the DTX-loading micelles, we chose the marketed formulation Taxotere® as the control. The DTX-loaded micelles were characterized by their core–shell structure, critical micelle concentration (CMC), particle size, zeta-potential, and the encapsulation efficiency of the micelle. The drug release, antitumor

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efficacy, pharmacokinetics and toxicity were compared for the two formulations.

2. Materials and methods

2.1. Materials

DTX was purchased from Norzer (Beijing, China). The $mPEG_{2000}$-DSPE was purchased from NOF Corporation (Tokyo, Japan). Taxotere® was obtained from Sanofi China (Shanghai, China). The HPLC-grade solvents used for high-performance liquid chromatography (HPLC) were purchased from Sayfo (Tianjin, China). All other chemicals were utilized as analytical grade preparations.

RPMI 1640 medium was purchased from M&C Gene Technology (Beijing, China). Trypsin and EDTA were purchased from Amresco (Solon, OH, USA). Fetal bovine serum (FBS) was purchased from Zhejiang Tianhang Biological Technology Co., Ltd. (Zhejiang, China).

2.2. Preparation and characterization of DTX-loaded micelles

The drug-loaded micelles were successfully prepared using the film formation method (Photos et al., 2003; Han et al., 2009). Briefly, DTX and $mPEG_{2000}$-DSPE (1:30, w/w) were dissolved in acetonitrile. The organic solvent was removed by rotary evaporation to form a film. The dried film was hydrated with 0.9% NaCl solution. The non-incorporated DTX was separated by filtration through a 220 nm poly(ether sulfone) membrane (Liu et al., 2008).

To show the core–shell micelle structure and drug loading characteristics of the DTX micelle, ^1H nuclear magnetic resonance (NMR) spectra were taken in $CDCl_3$ and D_2O using an Avance III 400 MHz Digital NMR Spectrometer (Bruker Corporation, Hamburg, Germany) (Li et al., 2009; Gill et al., 2012).

The critical micelle concentration (CMC) was estimated by the pyrene method (Opanasopit et al., 2007; Sezgin et al., 2006). A solution of 0.1 mg/mL pyrene in acetone (50 μL) was added to a 5 mL test tube and allowed to evaporate overnight to remove the acetone. A series of $mPEG_{2000}$-DSPE dissolved in normal saline (approximately 10^{-8} to 10^{-4} mol/L) were added to the test tubes containing pyrene to a concentration of 2.5 μg/mL. The mixtures were shaken in the absence of light for 24 h at room temperature. Experiments were set up with an emission wavelength of 390 nm, and the excitation spectra were scanned between 300 and 380 nm. The ratio of fluorescence intensity at 335 and 333 nm (I_{335}/I_{333}) was calculated and plotted against the logarithm of the concentration of micelles.

The size (hydrodynamic diameter) and zeta-potential of the micelles was measured by dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, United Kingdom). The particle size and zeta-potential measurements were carried out in triplicate. The morphological examination of the micelles was performed using a transmission electron microscope (TEM, JEOL, JEM-200CX, Japan). Briefly, a drop of micelle solution was placed on a copper grid and stained with 1% uranyl acetate solution and subsequently observed in the electron microscope.

The amount of DTX in the micelles was measured by HPLC analysis. After filtration through a 220 nm membrane, the micelle solution was diluted with acetonitrile, and the micelles were disrupted. The HPLC system was equipped with a UV detector (Waters Corporation, Milford, MA, USA) and a Dikma reverse-phase C18 column (4.6 mm × 150 mm). The column was eluted with methanol/acetonitrile/water (3/5/2, v/v/v) at 1.0 mL/min. DTX was detected at 232 nm. The encapsulation efficiency and the drug loading efficiency were calculated from the following equations:

$$\text{Encapsulation efficiency}\,(\%) = \frac{\text{weight of DTX in micelle}}{\text{weight of DTX fed initially}} \times 100\%$$

$$\text{Drug loading efficiency}\,(\%) = \frac{\text{weight of DTX in micelle}}{\text{weight of DTX in micelle} + \text{weight of lipid fed initially}} \times 100\%$$

The encapsulation efficiency and the loading efficiency tests were performed in triplicate.

2.3. Drug release in vitro

The drug release in vitro was performed in 0.5% Tween-80 phosphate-buffer saline (PBS, pH 7.4) at 37 °C (Liu et al., 2010). DTX micelle and Taxotere® equivalent to 0.7 mg of DTX were suspended in 0.7 ml of the relevant solvent in dialysis bags with a cut off M.W. 7000, which were placed in 100 mL 0.5% Tween-80 PBS, maintained at 37 °C and stirred at a speed of 200 rpm. At predetermined time intervals, 1 mL of the incubation medium was removed, and the same volume of fresh solution was added. The amount of DTX released at each time point was determined by an HPLC assay as described above. The experiments were carried out in triplicate.

2.4. In vitro antiproliferation assay

The human breast cancer cell line MCF-7 was cultivated in a humidified environment at 37 °C in a 5% CO_2 atmosphere. RPMI 1640 supplemented with 10% FBS and 1% penicillin–streptomycin was utilized as the cell culture medium. A sulforhodamine B (SRB) assay was used to evaluate the antiproliferation efficiency (Vichai and Kirtikara, 2006). Briefly, the cells were collected, counted and plated at a density of 13,000 cells/well in 96-well plates. The optical density (OD) of the cells was determined at a wavelength of 540 nm with a plate reader. The inhibition of proliferation by the drugs was calculated according to the formula (Fabbri et al., 2008) $[(OD_{treated} - OD_{zero})/(OD_{control} - OD_{zero})] \times 100\%$, in which OD_{zero} represents the number of cells before addition of the drug, $OD_{control}$ represents the number of cells in the untreated wells and $OD_{treated}$ represents the number of cells in the treated wells on the day of the assay. Each experiment was repeated three times.

2.5. Hemolytic evaluation

The hemolytic potential of the DTX micelle was determined using the method described by Ni et al. (2008). A 10 mL sample of fresh goat blood was collected in a 100 mL Erlenmeyer flask containing glass beads and shaken for approximately 10 min to remove the fibrinogen. Subsequently, the blood was diluted with decuple normal saline and centrifuged at 1500 rpm for 15 min. The supernatant was removed by pipette, and the precipitate was washed with normal saline 3 times. The precipitate was diluted to a suspension of 2% red blood cells (RBCs). Tubes (1.5 mL) were labeled from 1 to 7, and each number represented a set of 18 tubes. To all of the tubes, 200 μL of the RBC suspension was added. The tubes labeled No. 1 were diluted with 200 μL of distilled water as the hemolysis control, and the tubes labeled No. 2 were diluted with 200 μL of normal saline as the non-hemolysis control. The remaining sample tubes were diluted with 200 μL DTX micelle solutions of different concentrations. All of the tubes were

incubated in a 37 °C water bath. Three tubes from each number were dislodged at predetermined time intervals and centrifuged at 2000 rpm for 5 min. Then, 200 μL of supernatant was added into a 96-well plate. The absorbance was determined at 405 nm with an iMark microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). The hemolytic rate was calculated using the equation $(OD_{sample} - OD_{tube\ 1})/(OD_{tube2} - OD_{tube1})$. When this value was greater than 5%, the sample possessed hemolytic potential.

2.6. Pharmacokinetic studies

Female Sprague–Dawley (SD) rats (mean body weight 220 g, Vital River, Beijing, China) were used to perform the in vivo pharmacokinetic study. All rats were maintained in a light-controlled room kept at a temperature of 22 ± 2 °C and a relative humidity of 55 ± 5%. The experimental protocols involving animal study were approved by the Institutional Animal Care and Use Committee of Peking University.

The rats were randomly divided into a Taxotere® group or a micelle group and administered 8 mg/kg of DTX in Taxotere® or in micelle, respectively, via the tail vein. Blood samples (0.3–0.5 mL) were withdrawn from the ophthalmic vein at predetermined time intervals with heparinized tubes and centrifuged at 10,000 rpm for 5 min. Subsequently, 100 μL of plasma was added into a test tube and mixed with 10 μL of the internal standard (5 μg/mL paclitaxel solution). The sample was diluted with 2 mL of ether, and the mixture was vortexed for 5 min. The mixture was then centrifuged, and the organic phase was withdrawn and dried under a nitrogen stream at 40 °C. The dried residue was dissolved in 0.1 mL of methanol, and 50 μL of this sample was injected into the HPLC system. Pharmacokinetic parameters were obtained using the Practical Pharmacokinetic Program Version 97 (The Chinese Society of Mathematical Pharmacology).

2.7. In vivo antitumor efficacy and toxicity evaluation

The DTX micelles for evaluation of the in vivo antitumor efficacy and toxicity were prepared according to the method described in Section 2.2. The DTX concentrations were 0.5 mg/mL, 1 mg/mL and 2 mg/mL. The Taxotere® was prepared according to the operating manual, and the equivalent concentration of DTX was 1 mg/mL.

The antitumor efficacy studies were conducted in female Balb/c nude mice bearing MCF-7 cells (6–8 weeks old, approximately 21 g), which were purchased from Vital River Laboratories (Beijing, China). The animal experiments were approved by the Institutional Animal Care and Use Committee of Peking University.

For in vivo implantation, the MCF-7 cells were suspended in RPMI 1640 culture medium and injected subcutaneously at a concentration of 3×10^6 cells in 0.1 mL culture medium. When the tumor volume reached approximately 80 mm^3, the mice were randomly divided into five groups ($n = 8$), and that day was defined as day 0. These groups were injected through the tail vein with normal saline, 10 mg/kg Taxotere®, 5 mg/kg DTX micelle, 10 mg/kg DTX micelle or 20 mg/kg DTX micelle at day 0, 3 and 7. The tumor volumes ($1/2 \times [\text{major axis}] \times [\text{minor axis}]^2$) and body weights were measured for each animal every day. The relative tumor volume was calculated according to the formula V/V_0, where V represents the real-time tumor volume and V_0 represents the tumor volume on day 0 (Wang et al., 2009). The mice were euthanized at day 14, and the tumors were stripped, weighed and photographed.

The body weight variation was determined every day after day 0. On day 14, the body weights were acquired before the mice were euthanized, and the net weights of the mice were calculated as the body weight minus the tumor weight. Each body weight variation was calculated from the weight on each day minus the weight on day 0.

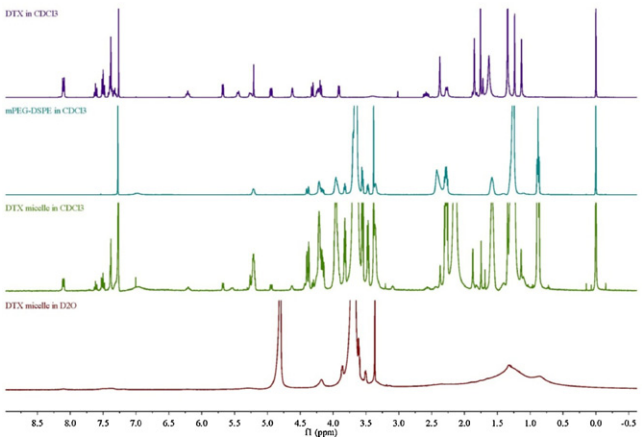

Fig. 1. From top to bottom, the ^{1}H NMR spectra of DTX, $mPEG_{2000}$-DSPE, the DTX-loaded micelle in $CDCl_3$ and the DTX-loading micelle in D_2O: 1.25 ppm, $-CH_2-$ in DSPE; 3.65 ppm, $-CH_2-O-$ in PEG; 7.30–7.63 ppm, benzene in DTX.

The myelosuppression effect was monitored using peripheral blood and bone marrow cells (BMCs) (Zhao et al., 2011). On day 14, blood samples were withdrawn from the ophthalmic vein and underwent the routine blood examination. After the mice were euthanized on day 14, a thigh bone was dissected from each mouse, and the cavum ossis was washed with 1 mL of PBS. The rinse solution was collected, and the BMCs were acquired by centrifugation. The BMCs were counted using a white blood cell counting chamber, and the cell cycles were analyzed by propidium iodide (PI) staining and flow cytometry.

2.8. Statistical analysis

Statistical analysis of the samples was performed using a one-way analysis of variance (ANOVA), and p-values < 0.05 were considered statistically significant. All data are reported as the means ± the standard deviation (SD) unless otherwise stated.

3. Results and discussion

3.1. Verification of the core–shell structure of the mPEG-DSPE micelle

The evidence for a core–shell type structure of $mPEG_{2000}$-DSPE micelles, limited mobility of the DSPE chain and drug loading in the core of the micelles was obtained using ^{1}H NMR in $CDCl_3$ and D_2O, as shown in Fig. 1. Because both the DSPE and PEG blocks are easily dissolved in $CDCl_3$, the core–shell structure was not expected, and the spectra of DTX micelles in $CDCl_3$ were composed of DTX spectra and $mPEG_{2000}$-DSPE spectra. However, the characteristic peaks of the DSPE blocks had obviously changed (peak type and peak strength) in D_2O because of the alteration in the chemical conditions, whereas the characteristic peaks of the PEG block (δ 3.65 ppm) remained. These results indicated that the protons of the DSPE block possessed restricted motion within the inner core and that the DSPE block had a rigid solid structure, whereas the PEG blocks existed in the liquid state in the aqueous environment. Additionally, drug entrapment within the inner core of the core–shell type micelles was confirmed with ^{1}H NMR in $CDCl_3$ and D_2O. The characteristic peaks of DTX disappeared in D_2O, whereas the peaks of PEG remained. However, in $CDCl_3$, both the characteristic peaks of the $mPEG_{2000}$-DSPE diblock copolymers and DTX appeared. These results clearly show that the hydrophobic drugs were successfully entrapped within the inner core of the core–shell

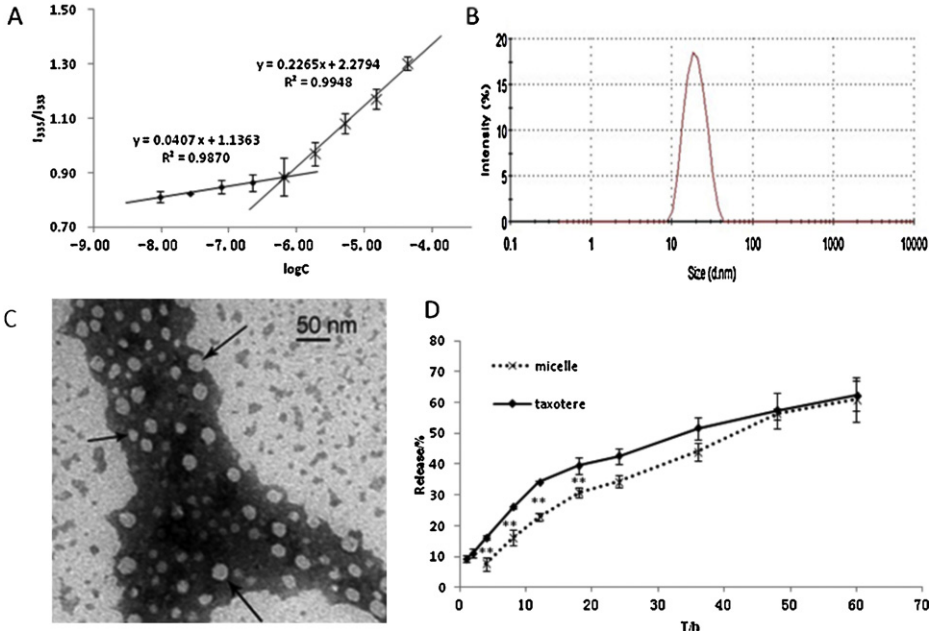

Fig. 2. Characteristics of the DTX-loading mPEG$_{2000}$-DSPE micelles. (A) Plot of the fluorescence intensity ratio of I_{335}/I_{333} of pyrene vs. the logarithm of the mPEG$_{2000}$-DSPE concentration in normal saline. (B) The size distribution of the micelles as determined by dynamic light scattering. (C) The morphology of the DTX-loading micelles by transmission electron microscope. (D) DTX release from the micelles and Taxotere®. The results are shown as the means ± SD ($n = 3$). ** represents that the released DTX from Taxotere® and micelles showed a significant difference ($p < 0.01$).

type micelles with a hydrated outer shell of PEG (Li et al., 2009; Gill et al., 2012).

3.2. Characteristics of the mPEG$_{2000}$-DSPE micelles

Pyrene was used as the fluorescence probe in the CMC determination because its fluorescence spectrum was sensitive to the polarity of the environment and shifted with increasing mPEG$_{2000}$-DSPE concentrations. The plot of the intensity of the I_{335}/I_{333} ratio of the pyrene against the logarithm of the polymer concentration was shown in Fig. 2A. The I_{335}/I_{333} ratio increased sharply when the polymer concentration reached the CMC. Thus, the intersection of the two fitting lines was just the CMC of mPEG$_{2000}$-DSPE, and this value was 7.04×10^{-7} mol/L. This result agreed with the value of $0.5–1 \times 10^{-6}$ mol/L that has been reported by Ashok et al. (2004). However, the CMC of mPEG$_{2000}$-DSPE reported by Lukyanov and Torchilin (2004) was 1.1×10^{-5} mol/L, and that of Johnsson et al. (2001) was 5.0×10^{-6} mol/L. Lukyanov and Torchilin explained that the pyrene method is one of the most sensitive and precise techniques for the determination of the CMC, and however, this method may not possess sufficient sensitivity for the measurement of the actual CMC values of amphiphilic compounds that contain blocks with double acyl chains. Thus, the determined CMC is "an upper estimation" rather than the actual value (Lukyanov and Torchilin, 2004). Even as an upper estimation, this value was much lower than that of most surfactants, including sodium dodecyl sulfate (SDS), tetradecyl trimethyl ammonium bromide (TTABr), polyoxyethylene-9-lauryl ether ($C_{12}E_9$) and the Tween series (Domínguez et al., 1997; Samanta and Ghosh, 2011). This result suggests that mPEG$_{2000}$-DSPE could easily form micelles in an aqueous environment and that these micelles may possess the ability to maintain their integrity even upon strong dilution in the body (Han et al., 2009; Lukyanov and Torchilin, 2004).

Because the size of the micelles is important for establishing drug delivery strategies to specific sites of the body, smaller micelles may tend to minimize the particle uptake by nontargeted cells, including their premature clearance by the mononuclear phagocytic system (MPS) (Brigger et al., 2002). The size of the drug-loaded micelles was determined to be 20.64 ± 3.79 nm by DLS (Fig. 2B), and they were spherical with a relatively monodisperse size (Fig. 2C) as determined by TEM. The zeta potential was 0.04 ± 0.08 mV, which indicates that the surface of the micelles is electrically neutral. Large molecules of more than 40 kDa in size and certain particles ranging from 10 to approximately 400 nm in size can leave the vascular bed and accumulate inside the interstitial space of the tumor, i.e., the EPR effect (Torchilin, 2011; Bae and Park, 2011). It is hypothesized that prepared micelles of appropriate size may be able to passively target the tumor site.

Many studies have reported that the major factor influencing both the loading capacity and efficiency of polymeric micelles is the compatibility between the solubilizate and the core-forming block (Mu et al., 2010). The drug loading efficiency of the mPEG$_{2000}$-DSPE micelle was 3.14 ± 0.13% and the encapsulation efficiency was 97.31 ± 2.95%. This result was caused by the hydrophobicity of DTX and the hydrophobic interaction between DTX and the DSPE core.

In the present study, the micelles were prepared from mPEG$_{2000}$-DSPE, which is highly compatible, and PEG was utilized as an external phase, which decreases the uptake of RES and enhances circulation in vivo. No additional toxic emulsifier was present throughout the preparation process. Therefore, the drug delivery system of mPEG$_{2000}$-DSPE micelles is expected to possess low toxicity.

3.3. Drug release in vitro

The percentage of drug release was plotted against time, as shown in Fig. 2D. The micelles possessed a lower and more stable drug release rate than Taxotere®. The release result revealed that the micelle was more stable when diluted and that the drug was slowly released from the micelle.

3.4. In vitro antiproliferation assay

A SRB assay was used to evaluate the IC_{50} values of the DTX micelle and the control, Taxotere®. The prepared micelle possessed an IC_{50} of 14.16 ± 4.52 ng/mL in cultured MCF-7 cells. The IC_{50} of

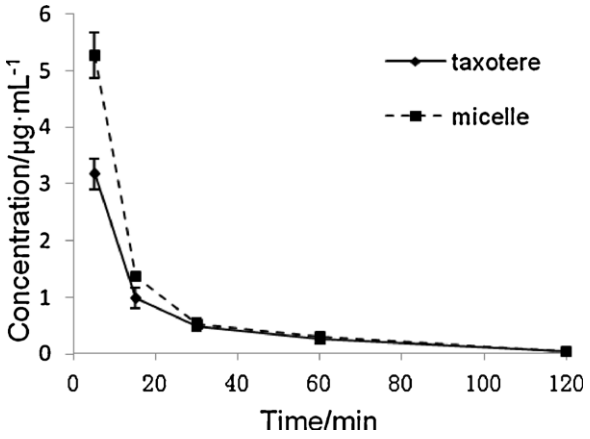

Fig. 3. The plasma concentration–time curves of DTX for the micelles and Taxotere®. The results are shown as the means ± SD. In the Taxotere® group, $n = 5$; in the micelle group, $n = 6$.

Taxotere® was 15.89 ± 6.38 ng/mL. Statistical analysis showed no significant difference between these two preparations.

3.5. Pharmacokinetics studies

The plasma concentration–time curves of DTX for the two formulations were shown in Fig. 3. The pharmacokinetic profiles of Taxotere® and the DTX micelle in rats were best described by a one-compartment model according to the Akaike Information Criterion (AIC). The pharmacokinetic parameters were summarized in Table 1. The DTX micelle showed a higher plasma concentration than Taxotere®, and the AUC of the DTX micelle was 1.5 times that of Taxotere®. This result indicated the micelle could help DTX avoid elimination and possess a long circulation effect in vivo. These results show the potential for enhanced antitumor efficacy and decreased toxicity in vivo.

3.6. In vivo antitumor efficacy

The relative tumor volume–time curve, tumor photographs and weight analysis were shown in Fig. 4. From the figure, we can make the following conclusions: (1) Mice in all of the groups that were administered DTX (Taxotere® or DTX micelle) had smaller tumors than the mice in the control group; (2) The DTX micelle was found to be more efficient at inhibiting tumor growth than Taxotere® for the comparison of the tumors of the 10 mg/kg DTX micelle group with those of the 10 mg/kg Taxotere® group. Statistical analysis revealed that there was a significant difference between the tumors of the two groups ($p < 0.05$), both in relative tumor volume and the weights of stripped tumors. The stronger antitumor efficacy of the micelles contributed to the passive targeting to tumor site i.e., the EPR effect (Kawano et al., 2006); (3) The DTX micelle showed increasing efficacy with increasing dosages in the range of 5–20 mg/kg. The tumors of mice in the 10 mg/kg DTX micelle group grew slowly over the 14 days after administration, while those of the 20 mg/kg DTX micelle group became smaller than they were at day 0.

Table 1
The pharmacokinetic parameters of DTX for the two formulations.

	Taxotere®	Micelle
Dose (mg/kg)	8	8
C_0 (μg/mL)	4.27 ± 1.23	8.70 ± 2.36
K_e (min^{-1})	0.08 ± 0.02	0.11 ± 0.03
V (L/kg)	1.87	0.92
$t_{1/2}$ (min)	8.67	6.30
AUC (μg mL/min)	53.43	79.09
Cl (L/kg/h)	0.15	0.10

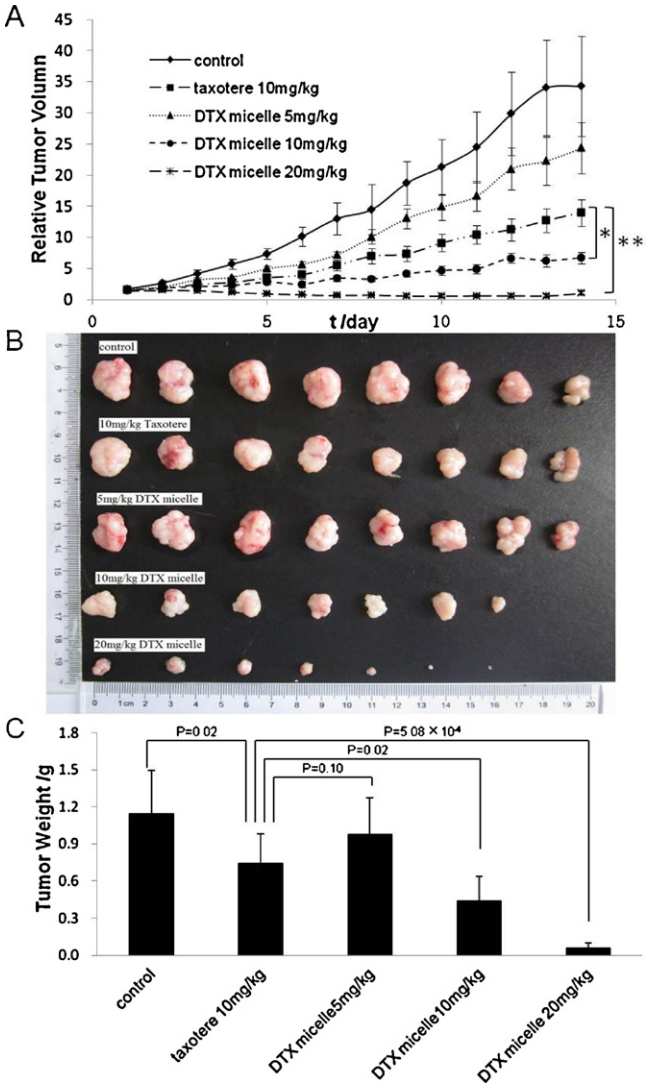

Fig. 4. The antitumor efficacy against mice bearing MCF-7. (A) Relative tumor volume–time curve. (B) Photograph of stripped tumors at day 14 after the mice were euthanized. (C) The weights of the stripped tumors at day 14 after the mice were euthanized. The results are shown as the means ± SE ($n = 7–8$). $^*p < 0.05$; $^{**}p < 0.01$.

3.7. Safety evaluation

The hemolytic potential of the DTX micelle was shown in Table 2. At all time points, the hemolytic rate was under 5%. This result indicated that the prepared DTX micelle had no hemolytic potential and may be safe for i.v. administration.

The variations in the body weights of the mice were plotted against time as shown in Fig. 5A, and the net weight variation at the conclusion of the experiment is shown in Fig. 5B. The body weights of the mice in the control group and the 5 mg/kg DTX micelle group gradually increased, but the body weights of the mice in the 10 mg/kg and 20 mg/kg DTX micelle groups and the 10 mg/kg Taxotere® group decreased. The net body weight results were consistent with the body weight variation–time curves. One-factor analysis of variance revealed no significant difference between the 10 mg/kg and 20 mg/kg DTX micelle groups and the 10 mg/kg Taxotere® group.

Table 2
The hemolytic potential (%) of the DTX micelle at different concentrations. The results are shown as the means ± SE (n = 3).

Time (min)	Concentration (mg/mL)				
	0.04	0.08	0.12	0.16	0.20
15	−2.40 ± 1.15	−3.11 ± 0.92	−2.86 ± 0.37	−3.56 ± 0.13	−4.14 ± 0.61
30	−2.93 ± 0.28	−2.29 ± 0.05	−2.56 ± 0.18	−2.90 ± 0.20	−3.65 ± 0.26
45	−2.42 ± 0.51	−2.74 ± 0.48	−2.07 ± 0.37	−2.38 ± 0.27	−1.43 ± 1.09
60	−1.21 ± 0.82	−2.42 ± 0.87	−2.38 ± 0.14	−2.17 ± 0.77	−2.17 ± 0.35
120	−3.13 ± 0.34	−4.05 ± 0.25	−3.78 ± 0.14	−3.63 ± 0.48	−2.05 ± 1.64
180	−3.08 ± 0.27	0.51 ± 3.85	−2.75 ± 0.64	−2.97 ± 0.19	−0.29 ± 0.98

Table 3
Routine peripheral blood tests and BMC counts of the mice in different groups. The results are shown as the means ± SD (n = 4–5).

	RBC ($\times 10^{12}$/L)	WBC ($\times 10^{9}$/L)	HGB ($\times$g/L)	PLT ($\times 10^{9}$/L)	GRN ($\times 10^{9}$/L)	BMC ($\times 10^{7}$/mL)
Control	7.16 ± 0.56	12.80 ± 4.25	122.2 ± 9.04	434.6 ± 91.09	10.10 ± 3.10	1.44 ± 0.42
10 mg/kg Taxotere®	7.04 ± 0.17	15.85 ± 2.64	123.8 ± 3.42	418.4 ± 67.76	14.54 ± 3.38	1.43 ± 0.30
5 mg/kg DTX micelle	6.70 ± 0.66	12.32 ± 2.02	120.2 ± 12.76	442.6 ± 106.73	9.82 ± 2.43	1.06 ± 0.24
10 mg/kg DTX micelle	6.94 ± 0.90	13.54 ± 3.58	122.4 ± 13.35	404.4 ± 66.73	10.58 ± 2.08	1.38 ± 0.26
20 mg/kg DTX micelle	6.89 ± 0.29	18.10 ± 5.57	120.4 ± 4.22	446.0 ± 74.84	15.28 ± 3.74	1.44 ± 0.28

The myelosuppression effects were evaluated by routine peripheral blood examination and BMCs. Increases in the WBC and GRN of the 20 mg/mL micelle and the 10 mg/mL Taxotere® groups were present, but the values were still within the tolerance range (Table 3). These results indicated that DTX did not promote myelosuppression in the dosage range of 5–20 mg/kg on day 14. We also analyzed the BMCs cycle, which may reflect latent myelosuppression (Table 4). Generally, as the number of cells in S phase increased, the potential for propagation increased. However, we found no significant difference in the cell numbers in S phase among all of the mice. Based on these results, we concluded that DTX and its preparations had no obvious myelosuppression effects in these dosage ranges.

On day 5 after treatment, the mice in the 10 mg/kg Taxotere® group and the 20 mg/kg DTX micelle group presented with many white flakes on their skin, while mice in the 10 mg/kg DTX micelle group had fewer of these flakes, and this phenomenon was not observed in mice in the control and 5 mg/kg DTX micelle groups. This dermatitis may be caused by the higher dose of DTX and the emulsifier of Taxotere® because Tween-80 is known to cause severe allergic reactions (Elsabahy et al., 2007; Liu et al., 2008).

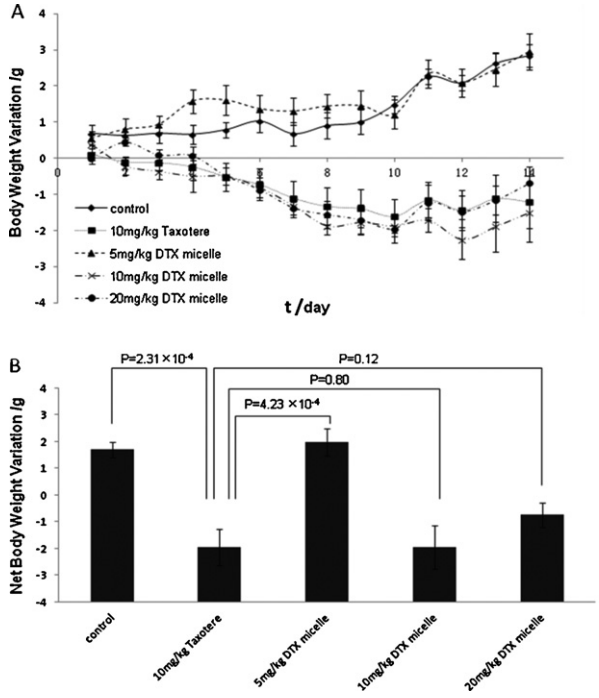

Fig. 5. The variation in body weight of the mice–time curves (A) and the variation in net weight at the conclusion of the experiment (B). The results are shown as the means ± SE (n = 7–8).

Table 4
The cycle distribution (%) of bone marrow cells for mice in different groups. The results are shown as the means ± SD (n = 7–8).

	G_0/G_1	G_2/M	S
Control	74.93 ± 1.64	4.48 ± 0.67	20.60 ± 1.51
10 mg/kg Taxotere®	75.39 ± 2.22	3.85 ± 0.53	20.77 ± 2.16
5 mg/kg DTX micelle	75.17 ± 2.15	4.48 ± 0.60	20.35 ± 2.21
10 mg/kg DTX micelle	76.77 ± 2.69	4.03 ± 0.14	19.20 ± 2.59
20 mg/kg DTX micelle	77.13 ± 3.55	4.50 ± 0.67	18.38 ± 3.00

4. Conclusion

In this work, a water-insoluble anticancer agent, DTX, was successfully incorporated into polymeric micelles formed from $mPEG_{2000}$-DSPE by the film formation method. The self-assembling properties of $mPEG_{2000}$-DSPE were studied by fluorescence analysis using pyrene as the fluorescent probe and ^{1}H NMR experiments. These techniques demonstrated that the $mPEG_{2000}$-DSPE copolymer is capable of self-assembly into micellar structures in water due to hydrophobic interactions between DSPE chains and the DTX was dissolved in the core of the self-assembled micelle. The prepared micelles were spherical with a hydrodynamic diameter of approximately 20 nm, which was confirmed by TEM and DLS analysis. The encapsulation efficiency was up to 97.31 ± 2.95%, which meets the requirement for administration in vivo. The prepared DTX micelle possessed an in vitro antitumor effect similar to that of Taxotere® and showed significantly improved in vivo efficacy over Taxotere® that may be caused by the passive targeting of the tumor by the micelle. In addition, the safety evaluation revealed the DTX micelle as a qualified drug to be utilized in vivo. Based on the experimental results, we conclude that the $mPEG_{2000}$-DSPE micelle may be a potent carrier for DTX.

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