**Enhanced tolerance and antitumor efficacy by docetaxel-loaded albumin nanoparticles**

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**Abstract**

Docetaxel is one of the most active chemotherapeutic agents for cancer treatment. The traditional docetaxel injection (TAXOTERE®) is currently formulated in the surfactant polysorbate 80, which has been associated with severe adverse reactions. To avoid the use of polysorbate 80 as well as to reduce the systemic toxicity of docetaxel, in this study, docetaxel-loaded albumin nanoparticles were fabricated by a novel simple self-assembly method. The resulting nanoparticles showed a mean diameter size of 150 nm. After being encapsulated into nanoparticles, docetaxel displayed similar cytotoxicity to traditional injection. Since polysorbate 80 was not involved in nanoparticles, the hemolysis was completely eliminated. The maximal tolerance dose of nanoparticles was also increased, which allowed a higher dose to be safely intravenously injected and produced ideal antitumor effects. The 150 nm diameter also allowed the nanoparticles to accumulate in tumor tissue via the enhanced permeability and retention effect. The passive targeting ability further caused the higher antitumor effects of nanoparticles than that of traditional injection at the same dose (7.5 mg/kg). Therefore, docetaxel-loaded albumin nanoparticles fabricated by our strategy showed higher promise in their safety and effectiveness than the traditional docetaxel injection.

**Keywords**

Albumin; cancer chemotherapy, drug delivery system, nanoparticles, self-assembly

**Introduction**

Docetaxel (DTX), a semi-synthetic taxane, is a promoter of microtubule polymerization leading to cell cycle arrest at G2/M, apoptosis and cytotoxicity (Ringel & Horwitz, 1991). It has significant activity in the treatment of breast, non-small-cell lung, ovarian, and head and neck cancers (Clarke & Rivory, 1999). Because of the bulky, extended fused ring with several hydrophobic substitutes in chemical structure, DTX shows higher promise in their safety and effectiveness than the traditional docetaxel injection.

The traditional DTX injection (TAXOTERE®) for cancer chemotherapy was initially approved by the Food and Drug Administration (FDA) in 1996, which is formulated as 40 mg/mL solution in the non-ionic surfactant polysorbate 80 (polyoxyethylene sorbitan monooleate). It is diluted with a three-fold volume solvent of ethanol and water (13:87, v/v) before further dilution in infusion fluid (Nuijen et al., 2001; Desai et al., 2010; Jiang et al., 2015). Up to now, all three formulations of DTX approved by the FDA are using polysorbate 80 as adjuvant (25% in TAXOTERE®, 32% in DOCEFREZ®, 8% in DOCETAXEL®, described in product information) (Drugs@FDA, 2014). However, polysorbate 80 has been reported in the contribution of acute hypersensitivity reactions and peripheral neuropathy (ten Tije et al., 2003). Significant hemolytic activity has also been reported due to polysorbate 80 (Cheon Lee et al., 2003; Quaglia et al., 2009). In addition, polysorbate 80 can inhibit the binding of taxanes to albumin (De et al., 2005), hence inhibiting the albumin-based drug transport (Desai et al., 2006b; Desai, 2007).

Therefore, investigation of alternative formulations of DTX without polysorbate 80 is an ongoing research. Several approaches based on liposomes (Immordino et al., 2003; Liu et al., 2010; Muthu et al., 2012; Tong et al., 2012; Kutty & Feng, 2013), vesicles and polymer nanoparticles (Upadhyay et al., 2010; Leonhard et al., 2012; de Oliveira et al., 2013) have been proposed. However, all these strategies failed in preclinical research or were terminated in clinical trials (Duncan, 2003; Ferrari, 2005). One of the reasons for the failure is possibly due to the materials themselves, such as lipid and synthetic polymer, which cannot be normally degraded in body, and present toxicity risks (Rihova, 1996; Peer et al., 2007; Davis et al., 2008).

In this case, protein-based nanoparticles which employ endogenous plasma proteins, especially albumin, come to the best option of drug vectors (Maham et al., 2009; Elzoghby et al., 2012). Besides the traditional passive tumor targeting via the enhanced permeability and retention (EPR) effect arisen from the abnormalities in vasculatures in tumor
(Cho et al., 2008), active targeting is also integrated by taking albumin as drug vectors. Albumin-based nanoparticles have been shown to selectively accumulate in tumor tissues because of its high affinity with albumin receptors over-expressed on tumor cell surface, such as gp60 and secreted protein, acidic and rich in cysteine (SPARC) (Desai et al., 2006b; Desai, 2007). Therefore, albumin could be an ideal vector for delivering DTX.

At present, albumin nanoparticles loading DTX (ABI-008) have been successfully fabricated (Desai et al., 2006a) and entered into phase II clinical trial (Desai et al., 2006a; Hawkins et al., 2008). This nanoparticle albumin-bound (nab™) technology is considered to be the most effective (Desai, 2007; Fu et al., 2009). However, many problems still exist. Firstly, the fabrication method is too complex to be carried out in industrial scale production (Desai et al., 2010). Besides, toxic organic solvent chloroform is used in nanoparticle preparation, which cannot be removed completely. A certain amount of residual chloroform may lead to high toxicity in liver, kidney, lung and neural system (Desai et al., 2010; Bale et al., 2011). Also, phase II clinical trial of albumin nanoparticles loading DTX for the treatment of metastatic breast cancer has been terminated (ClinicalTrials.gov, 2014), which may be due to the high toxicity. Therefore, it is necessary to develop a new method to prepare DTX-loaded albumin nanoparticles.

To overcome above-mentioned barriers, we had successfully pioneered a novel simple strategy to fabricate drug-loaded albumin nanoparticle in our previous work (Gong et al., 2011; Hu et al., 2011; Gong et al., 2012). In this strategy, albumin was unfolded to expose hydrophobic regions and interacted with hydrophobic drugs to form nanoparticles. This self-assembly method eliminated the complicated preparation procedures and toxic residual solvents. It maintained a mild process condition for protein and drug, by which the prepared nanoparticles were proven to be highly effective in antitumor activity (Ding et al., 2014). In this study, we prepared DTX-loaded albumin nanoparticles (DTX-NPs) by our strategy. The general adaptability of our technology in hydrophobic drugs was testified, and some novel fabrication parameter details were readjusted. The physicochemical features, in vitro stability and release of DTX-NPs were characterized. We also reported that, compared to DTX injection, DTX-NPs showed reduced side effects and increased antitumor efficacy.

Materials and methods

Materials

Docetaxel (DTX) was obtained from Sunve Pharmaceutical Co., Ltd. (Shanghai, China). Human serum albumin (HSA) was produced by Baxter AG (Vienna, Austria). Dithiothreitol (DTT) was purchased from SunshineBio Co., Ltd (Nanjing, China). Docetaxel injection was manufactured by Hengrui Medicine Co., Ltd (Lianyungang, China). Other reagents were purchased from Sigma-Aldrich (St. Louis, MO). Cell-Counting Kit-8 (CCK-8) was obtained from Dojindo Laboratories (Kumamoto, Japan). NIR-775 was bought from Sigma-Aldrich (St. Louis, MO). Other reagents were purchased from Aldrich (St. Louis, MO). Mouse colon carcinoma cell line CT26 and human lung adenocarcinoma epithelial cell line A549 were cultured by State Key Laboratory of Pharmaceutical Biotechnology (Nanjing, China). BALB/c mice (6–8 weeks age and weighing 18–22 g) and rabbits were provided by Qinglongshan Experimental Animal Center (Nanjing, China). All studies were carried out in accordance with the Institutional Animal Care Committee at the Nanjing University.

Preparation of DTX-NPs

Human serum albumin was dissolved in distilled water (4 mg/mL) and DTT was added with stirring. Then DTX dissolved in ethanol (60 mg/mL) was slowly added. Thus, the solution changed into pale-blue, indicating the formation of nanoparticles. The sample was dialyzed in water with 10 kDa MWCO dialysis membranes to remove DTT, filtered with 0.22 μm filters, and then freeze dried for storage.

Size, zeta potential and morphology of DTX-NPs

The mean diameter, size distribution and zeta potential of DTX-NPs were measured using Zetasizer Nano (ZN3600, Malvern, Worcestershire, UK). The sizes were determined by dynamic light scattering (DLS) and the potentials were determined by electrophoretic light scattering (ELS). Samples were diluted with water to a protein concentration of 2 mg/mL before the measurements of triplicate.

Transmission electron microscopy (TEM, H-7650, Hitachi, Tokyo, Japan) and scanning electron microscopy (SEM, S-3400N, Hitachi, Tokyo, Japan) were utilized to investigate the morphology of DTX-NPs. Before TEM observation, samples were dissolved in water and dried overnight on a copper grid. For SEM examination, reconstituted samples were dispersed in 0.65% glycerol, spread on a cover slide, immobilized in 2% formaldehyde and dehydrated in a graded serial of ethanol–water solutions. The specimen surfaces were coated with platinum before being subjected to SEM.

Drug loading content and encapsulation efficiency of DTX-NPs

Twenty milligrams of DTX-NPs lyophilized powder was reconstituted by 10 mL distilled water. The dispersion was diluted in 10-fold volume of acetonitrile [high-performance liquid chromatography (HPLC) grade, Fisher Scientific, Fair Lawn, NJ], vortexed for 1 min and centrifuged at 12,000 g for 10 min (Centrifuge 5430, Eppendorf, Hamburg, Germany). Then, the supernatant was injected into an HPLC system with a C18 column (250 × 4.6 mm, 5 μm, Agilent, Santa Clara, CA) for DTX analysis. The mobile phase consisted of 0.043 M ammonium acetate solution and acetonitrile (45:55, v/v). The flow-rate was set as 1.0 mL/min, while the UV detection wavelength was 232 nm. The drug loading content was calculated by Equation (1):

\[
\text{Drug loading content (\%) = \frac{\text{Amount of DTX in DTX-NPs}}{\text{Amount of DTX-NPs}} \times 100\%}
\]

Therefore, the DTX-NPs were passed through a column of Sephadex G-50 (20 cm × 1.1 cm). HSA-encapsulated DTX was separated from free DTX by washing the column with water and ethanol successively. The collected fractions were
analyzed by the same HPLC procedure. The encapsulation efficiency was calculated by Equation (2):

\[
\text{Encapsulation efficiency (\%) = \frac{\text{Amount of encapsulated DTX}}{\text{Amount of (encapsulated DTX + free DTX)}} \times 100\%}
\]

Status of DTX and HSA in DTX-NPs

To determine the crystalline or amorphous status of DTX in DTX-NPs lyophilized powder, X-ray powder diffraction (XRD) was employed. The following samples were analyzed: DTX crystal powder, HSA powder, physical mixture powder of 9:100 DTX/HSA (w/w) and DTX-NPs powder loading with 9:100 DTX/HSA (w/w). They were examined under an X-ray diffractometer (XTRA/3 KW, ARL, Ecublens, Switzerland) after fully grinded, while the incidence angle 2θ was from 3° to 50° and the wavelength was 1.5405 Å.

Also, we utilized circular dichroism (CD) spectrum to explore the change of secondary structure status of HSA before and after the manufacture of DTX-NPs. HSA and DTX-NPs were diluted with water to a protein concentration of 50 μg/mL and subjected to analysis by a CD Spectropolarimeter (J-810, Jasco, Tokyo, Japan). The molar ellipticity was measured at the wavelength from 180 to 400 nm in triplicate. Then, respective proportions of different secondary structures including helix, sheet, turn and random coil were calculated by according to the method by Yang et al. (1986).

In vitro stability of DTX-NPs

DTX-NPs were analyzed for stability at different suspension conditions. We reconstituted samples in 0.9% sodium chloride before and after the manufacture of DTX-NPs. HSA and DTX-NPs were analyzed for stability at different suspension conditions. We reconstituted samples in 0.9% sodium chloride before and after the manufacture of DTX-NPs. HSA and DTX-NPs were diluted with water to a protein concentration of 50 mg/mL. In the following time points, 1, 2, 4, 8, 12, 24 and 48 h, mean particle diameter changes were measured by DLS.

In vitro release of DTX-NPs

The in vitro release of DTX-NPs was also investigated. 0.2 M Phosphate buffer, pH 7.4, containing 2% β-cyclodextrin (m/v) was used as release medium. DTX-NPs and DTX injection (containing 2 mg of DTX) were added into dialysis tubes (10 kDa MWCO) and dipped in beaker flasks containing 200 mL release medium, respectively. These flasks were placed in a shaking bath at 37°C with a constant agitation of 100 strokes/min. At the time points of 1, 2, 4, 8, 12, 24 and 48 h, the amounts of DTX in the medium samples were determined by HPLC.

In vitro cytotoxicity of DTX-NPs

The in vitro cytotoxicity assay of DTX-NPs and DTX injection were performed on human breast adenocarcinoma cell line MCF-7 and mouse colon carcinoma cell line CT26. In particular, 3000 cells/well were seeded in 96-well plates (Costar, Washington, DC) and then incubated for 12 h at 37°C for cell adhesion. Then, 100 μL of a serial concentration of DTX-NPs and DTX injection were added to each well, with 100 μL phosphate-buffered saline (PBS) in medium as control, and incubated for another 48 h. On the following day, 10 μL of cell counting kit-8 (CCK-8) was added into each well, and cells were incubated for another 2 h. Then, the solution was measured at 450 nm on a microplate reader (Safire, Tecan, Männedorf, Switzerland). Based on the optical density (OD) values, the cell viabilities were evaluated.

In vitro hemolysis of DTX-NPs

Rabbit blood was used for evaluating the hemolysis of DTX-NPs in comparison with DTX injection. The fresh blood was washed with normal saline, centrifuged at 120 g for 10 min, and then the supernatant was discarded. The red blood cells (RBC) suspensions were diluted with normal saline to a concentration of 2% (v/v). 1.5 mL DTXNPs and DTX injection with DTX concentration range from 0.01 to 1 mg/mL were added to 1.5 mL RBC suspensions individually (n = 3). After incubation at 37°C for 30 min, the mixture was centrifuged at 3000g for 10 min to remove nonlysed RBC. The supernatant was collected and analyzed for released hemoglobin by ultraviolet spectrophotometer (UV-2450, Shimadzu, Kyoto, Japan) at 570 nm. In addition, to obtain 0 and 100% hemolysis, 1.5 mL normal saline and 1.5 mL distilled water were added to 1.5 mL RBC suspensions, respectively. The hemolysis ratios were calculated by Equation (3), where Abs, Abs0 and Abs100 are the absorbencies of the samples, 0% hemolysis solution and 100% hemolysis solution, respectively.

\[
\text{Hemolysis} \% = \frac{\text{Abs} - \text{Abs0}}{\text{Abs100} - \text{Abs0}} \times 100\%
\]

In vivo tolerance of DTX-NPs

A comparison of in vivo tolerance in mice between DTXNPs and DTX injection were investigated. Male BALB/c mice (n = 3) were tail intravenously injected with various doses of DTXNPs and DTX injection, which were diluted with normal saline, respectively. A daily dose of 40 mg DTX/kg body was given and the final cumulative dose was recorded. The survival of each group was also analyzed.

In vivo NIRF imaging of DTX-NPs

Near infra-red fluorescent system (NIRF) was applied to explore the real-time in vivo biodistribution of DTX-NPs. Particularly, DTX-NPs were labeled by a NIRF dye, NIR-775. In the preparation process, NIR-775 and DTX (1:9 NIR-775:DTX, wt/wt) were dissolved in ethanol and co-assembled into nanoparticles (NIR-775-DTX-NPs). Meanwhile, CT26 tumor bearing model was developed by subcutaneously inoculating CT26 cells to male BALB/c mice at the right axilla. Then, 0.25 mg/kg (calculated as NIR-775) of NIR-775-DTX-NPs and free NIR-775 were injected into tumor bearing mice via the tail vein. Free NIR-775 was dissolved in polysorbate 80 and diluted with three-fold volume of 13% ethanol. At the time intervals of 2, 4, 8, 12, 24, 28 and 32 h after the administration, the biodistributions were imaged utilizing the IVIS® Lumina System (Xenogen, Alameda, CA). The excitation wavelength was 745 nm, the exposure time was set as 1 s, and the NIRF images at 800 nm were collected.
In vivo tissue distribution of DTX-NPs

The in vivo tissue distribution of DTX-NPs was evaluated compared with DTX injection in human tumor xenograft model. Human lung adenocarcinoma epithelial cells, A549, were subcutaneously inoculated to male BALB/c nude mice at the right axilla. The mice with tumor were selected randomly and equally divided into two groups (n = 3) as subjects. Two formulations, DTX-NPs and DTX injection were administered to the two groups at a 10 mg/kg dose via the tail vein, respectively. Then the mice were euthanized at 24 h, and the tumor, heart, liver, spleen, lung, kidney were collected, washed, weighed and homogenized (T25 Ultra-Turrax Homogenizer, IKA, Baden-Wurttemberg, Germany) in 1 mL saline. 200 µL tissue homogenates was extracted by adding 250 µL methanol and 250 µL acetonitrile vortex-mixing the samples for 30 s. The mixture was then centrifuged for 15 min at 30 000 g, and the supernatant was transferred, filtered and injected into the HPLC system with paclitaxel as internal standard to determine DTX.

In vivo antitumor efficacy of DTX-NPs

The in vivo antitumor efficacy of DTX-NPs and DTX injection were investigated in the A549 xenograft mice model. The tumor volumes (V) were calculated as \( V = \frac{d^2 \times D}{2} \), where d and D were the shortest and the longest diameters of the tumor in mm. When the volumes reached about 150 mm³, mice were divided into five groups (n = 7) and the treatments started. The treatment groups were administered intravenously with DTX-NPs at DTX doses of 7.5 and 30 mg/kg, DTX injection at DTX doses of 5 and 7.5 mg/kg, three times at 7-day intervals, respectively. The control group was given normal saline only. Afterwards, the mean tumor volumes were measured every day.

Statistical analysis

Results were presented as the mean ± standard deviation if there is no special explanation. Data were analyzed by one-way analysis of variance. Probability values <0.05 were considered to be significant.

Results and discussion

Preparation of DTX-NPs

In our previous work, we successfully developed a novel strategy to prepare drug-loaded albumin nanoparticles (Gong et al., 2011, 2012; Hu et al., 2011). This method is based on the hydrophobic interactions between drugs and internal regions of protein. By analyzing the crystal structure of albumin, a few scattered hydrophobic binding sites were observed on the molecular surface but large hydrophobic cavities hide in the inner protein molecule, and the protein framework is maintained by 17 disulfide bonds (Sugio et al., 1999; Paal & Shkarupin, 2007; Kratz, 2008). By reducing disulfide bonds with β-mercaptoethanol, the hydrophobic regions are exposed and will interact with hydrophobic drugs resulting in nanoparticles. In this study, DTX was taken as hydrophobic drug and mixed with DTT and HSA to form nanoparticles. This method avoids the complexity of preparation procedure and the toxicity of residual solvents as well as maintains a mild process condition for protein and drug. The disulfide bonds reductant in our previous method, β-mercaptoethanol, was replaced by dithiothreitol. Dithiothreitol has low toxicity and little pungent odor, and can be used at a much lower concentration than β-mercaptoethanol (Konigsberg, 1972). This improvement is significant for industrialization produce and clinical use.

Size, zeta potential and morphology of DTX-NPs

To explore the influence of the initial ratio of added drug/protein on particle property, a series of ratios from 0 to 30% (w/w, DTX/HSA) were used to prepare DTX-NPs (Table 1).

When no drug was added into protein (0%), the particles existed mainly in the form of albumin monomer or multimer with the mean diameter of around 15 nm. Along with the ratio increase, no significant increase in particle size was observed. When the ratio was up to 15%, nanoparticles of about 150 nm size were formed. Furthermore, the mean diameter stayed stable in the range of 15–20%. This size range makes nanoparticles easy to accumulate in tumors due to the EPR effect (Cho et al., 2008). When the ratio exceeded 20%, the particle size continued to increase. At 25%, the formed particles showed a diameter of 183 nm. When the ratio further increased, unstable nanoparticles were observed, which might be due to the fact that hydrophobic sites on protein were not enough to accommodate the added drugs. In all particles with various drug/protein ratios, their zeta potential stayed constant at about −20 mV (Table 1). This might be due to the fact that the pH of the preparation solution was above the isoelectric point of albumin, giving negative charges to nanoparticles. This negative charge guarantees the stability of nanoparticles in aqueous solution, such as NaCl and glucose.

In this study, DTX-NPs were prepared by adding DTX and HSA at a ratio of 18% (w/w) if there is no particular mention. The DLS measurement indicated the formation of DTX-NPs with a relatively narrow size distribution (polydispersity = 0.053) (Figure 1a) and zeta potential of −20 mV (Figure 1b). The representative morphology images of DTX-NPs were also observed. In the TEM and SEM images, DTX-NPs had a spherical shape and smooth surfaces (Figure 1c and d). The particle size is in good agreement with that detected by DLS.

| Table 1. The size and Z-potential of DTX-NPs with different ratios of DTX/HSA. |
|-----------------|-----------|----------------|
| DTX/HSA (w/w)   | Size (nm) | Z-potential (mV) |
| 0%              | 11.2 ± 1.4 | −20.1 ± 0.9    |
| 5%              | 16.2 ± 3.8 | −19.8 ± 1.8    |
| 10%             | 21.5 ± 3.7 | −20.5 ± 1.5    |
| 15%             | 145.4 ± 11.3 | −18.4 ± 0.7  |
| 18%             | 146.5 ± 11.7 | −17.3 ± 2.9   |
| 20%             | 141.7 ± 16.9 | −21.9 ± 1.2   |
| 25%             | 183.0 ± 20.9 | −20.8 ± 0.5   |
| 30%             | 365.9 ± 39.8 | −17.5 ± 2.2   |
Drug loading content and encapsulation efficiency of DTX-NPs

The drug loading content increased with the increase of the initial ratio of added drug to protein. The drug loading of DTX-NPs reached 8.33% as the initial ratio of added DTX to HSA was 18%. The divergence between drug loading and the ratio of added drug to protein can be attributed to the loss of DTX in the preparation process. Although DTX is generally known as a hydrophobic agent, it still shows higher watersolubility than other similar drugs, which results in a certain amount of free drug dissolution in water and loss at the dialysis stage. We also evaluated the encapsulation efficiency of DTX-NPs. As shown in Figure 2, DTX-NPs were first eluted out of column and exhibited a milky color. Free DTX was subsequently eluted out of column by ethanol and exhibited a clear solution. The calculated encapsulation efficiency was 98.33%, which meant nearly all drugs were encapsulated into HSA nanoparticles.

Status of DTX and HSA in DTX-NPs

The crystal state of DTX existing in DTX-NPs was observed in comparison with free DTX, HSA and their physical mixture. As the XRD patterns shown in Figure 3(a), free DTX demonstrated strong characteristic diffraction peaks at 4°, 10° and 18°, suggesting it was crystalline. Similar diffraction peaks could also been observed in the physical mixture of DTX and HSA, suggesting DTX in the mixture was still crystalline. DTX-NPs did not display those characteristic diffraction peaks, which indicated DTX was amorphous in DTX-NPs. The amorphous DTX is a readily bioavailable state and is beneficial to be utilized by body after intravenous administration (Desai, 2007).

The secondary structure of HSA in DTX-NPs was also investigated. In the CD spectrum, there were some biases in far ultraviolet range from 180 to 250 nm for DTX-NPs compared with natural HSA. We estimated the proportions of secondary structures of HSA by using Yang’s spectra as reference (Yang et al., 1986), including $\alpha$-helix, $\beta$-sheet, $\beta$-turn and random coil. As shown in Figure 3(b), $\alpha$-helix decreased while $\beta$-turn increased. No significant changes were observed in $\beta$-sheet and random coil. The changes in $\alpha$-helix and $\beta$-turn indicate the potential changes in albumin conformation.

In vitro stability of DTX-NPs

To evaluate the stability of DTX-NPs, the changes of particle size were recorded in different solutions including 0.9% NaCl.
and 5% glucose. The mean particle size of DTX-NPs remained constant at about 150 nm after 48 h at 25 °C (Figure 4a). No precipitate was observed during the whole process. Commonly, the stability of DTX injection (TAXOTERE®) lasts only for 4 h and it begins to precipitate out of solution after 4 h (Sanofi-Aventis, 2014). This suggests DTX-NPs are more stable than DTX injection. The stability is one of the most crucial factors for clinical use of drug formulation since the drug may slowly escape from nanoparticles and form secondary aggregates, thereby possibly leading to the blood vessel occlusion (Wang et al., 2010). The high stability also ensured DTX-NPs a long circulation time in vivo, an increased accumulation in tumors and better antitumor efficacy (Venkatraman et al., 2010).

**In vitro release of DTX-NPs**

The in vitro release profile of DTX-NPs was also evaluated. In the medium containing 2% β-cyclodextrin (m/v), DTX-NPs showed a two-phase release which includes a rapid release in the first 4 h and a more sustained release for the following 44 h (Figure 4b). The total cumulative release was 91.5% in the end. As control groups, DTX injection showed a burst release in the first 4 h and the total cumulative release reached the maximum (91.0%) in 8 h. Compared with the low accumulative release rate in our previous trials with
polysorbate 80 or sodium dodecyl sulfate medium, β-cyclodextrin could carry more released DTX out of the dialysis tubes for analysis. Although DTX-NPs showed high particle-stability in static NaCl and glucose solution, DTX released from nanoparticle as sufficiently as traditional injection in this flowing medium with an adequate holding capacity. The nearly 24 h for approaching the top cumulative release confirmed that DTX-NPs could achieve a long circulating effect. This slow and continuous release of DTX can reduce the peak concentration and prolong the therapeutic drug level (Venkatraman et al., 2010).

**In vitro cytotoxicity of DTX-NPs**

To investigate the cytotoxicity of DTX-NPs, human breast adenocarcinoma cell line MCF-7 and mouse colon carcinoma cell line CT26 were used. As shown in Figure 5, a dose-dependent cytotoxicity was observed both in MCF-7 and in CT26. With the increase of concentration, the cytotoxicity of DTX-NPs increased. Ranging from 0.008 to 8000 ng/mL, DTX-NPs displayed relatively same significant cell inhibition as DTX injection, indicating an equivalent cytotoxicity. Based on these results, we speculate that DTX-NPs can obtain the pharmacological efficacy as similar as DTX injection.

**In vitro hemolysis of DTX-NPs**

As polysorbate 80 has to be used in DTX injection, hemolysis is a common result. In DTX-NPs, DTX was encapsulated by albumin and no polysorbate 80 was involved. As shown in Figure 6, less hemolysis was observed in DTX-NPs. In DTX

![Figure 5. In vitro cytotoxicity of DTX-NPs. (a) Cell viability of MCF-7 against DTX injection and DTX-NPs for 48 h co-incubation; (b) Cell viability of CT26 against DTX injection and DTX-NPs for 48 h co-incubation. Each data point is represented as mean ± SD (n = 6).](image)

![Figure 6. In vitro hemolysis of DTX-NPs. (a) The hemolysis percentage of DTX-NPs and DTX injection. Each data point is represented as mean ± SD (n = 3). (b) The hemolysis photograph of DTX-NPs and DTX injection.](image)

![Figure 7. In vivo tolerances of DTX-NPs. Survival of mice after injection of series cumulative doses of DTX-NPs and DTX injection.](image)
injection, 0.05 mg/mL caused severe side effects. As the concentration increased, more serious hemolysis was observed in DTX injection but it was scarcely observed in DTX-NPs. Even at 1.0 mg/mL, DTX-NPs only showed 10% hemolysis while DTX injection causing 86% hemolysis. Therefore, in terms of hemolysis, DTX-NPs are much safer than DTX injection.

**In vivo tolerance of DTX-NPs**

To compare the tolerance in vivo, we also evaluated the survival of mice after injecting a series of cumulative dose of DTX-NPs and DTX injection. As shown in Figure 7, dead mice were observed at 40 mg/kg of DTX injection and all mice died when the cumulative dose reached 160 mg/kg. No dead mice were observed even after the cumulative dose reached 200 mg/kg in DTX-NPs. This significant difference between two DTX formulations might be due to the elimination of adjuvant polysorbate 80 and reduction of the systemic toxicity in DTX-NPs. These results prove that DTX-NPs are well-tolerated, indicating higher potential safety in clinical use.

**In vivo imaging of NIR dye-loaded DTX-NPs**

To explore the real-time biodistribution of DTX-NPs, NIR-775 and DTX was co-encapsulated into albumin nanoparticles (NIR-775-DTX-NPs). NIR-775 is a NIR dye which has been used for NIR imaging extensively (Ex = 745 nm, Em = 800 nm). It has deep tissue penetration with low tissue absorption and scattering (Peng et al., 2006). The resulting nanoparticles (NIR-775-DTX-NPs) showed the same characteristics as DTX-NPs (data not shown). As shown in Figure 8(a), high intensity of fluorescence was detected in the liver and the tumor area at 2 h after administration of NIR-775-DTX-NPs. Compared to normal tissues, the fluorescence intensity in the tumor was gradually enhanced with time elapsed and it lasted for more than 24 h after injection. After the administration of free NIR-775, although high intensity of fluorescence in liver area was detected, the fluorescence in tumor area was low. These were also demonstrated by the calculated percentages of tumor accumulation. As shown in Figure 8(b), over 20% of fluorescence accumulated in tumor after the injection of NIR-775-DTX-NPs, and the level continued growing even after it exceeded 40% at 24 h. On the contrary, the percentage was less than 20% after the injection of free NIR-775, and the level became to decline after it meagerly reached to 25% at 24 h. This tumor accumulation indicates the in vivo targeting ability of DTX-NPs, which might be due to the EPR effects (Cho et al., 2008) and the SPARC pathway (Desai et al., 2006b; Desai, 2007).
In vivo antitumor efficacy of DTX-NPs

To evaluate the in vivo antitumor efficacy of DTX-NPs, A549 xenograft mice were intravenously injected with DTX-NPs, DTX injection, and saline three times at 7-day intervals, respectively. According to the high tolerance of DTX-NPs, 7.5 mg/kg and 30 mg/kg were chosen for antitumor evaluation. Since death occurred at 10 mg/kg of DTX injection in our pre-experiment, the dose for DTX injection was set as 5 and 7.5 mg/kg. Figure 9(a) demonstrated the tumor growth curves. In the saline control group, the tumor volume increased rapidly from the initial average volume of about 150 to 1403.60 mm³ on day 22. In DTX injection groups, the tumor increased greatly, allowing a higher dose to be safely intravenously injected. In addition, at the same dose (7.5 mg/kg), the antitumor effects of DTX-NPs were higher than that of DTX injection, which might be due to the passive targeting ability of DTX-NPs to tumor tissue. Therefore, DTX-NPs can be highly efficiently transported into tumor via gp60 receptors. (3) Because polysorbate 80 is not involved in DTX-NPs, the toxic effects caused by polysorbate 80 are eliminated and the maximal tolerance dose of DTX is also significantly increased. Thus, significant antitumor effects can be obtained.

Conclusion

In summary, by using the self-assembly method developed by our group, we successfully encapsulated DTX into HSA to form nanoparticles. These DTX-NPs avoided the adjuvant polysorbate 80 with potential side effects in traditional DTX injection, and eliminated the complicated preparation procedure and toxic residual solvents in common preparation method. By using the simple self-assembly method, 150 nm DTX-NPs were produced and the desirable drug loading content and high encapsulation efficiency were also achieved. The resulting DTX-NPs developed by the method displayed high stability in suspension solutions, and displayed similar cytotoxicity to DTX injection. One of the most serious side effects induced by polysorbate 80, hemolysis, was completely eliminated. The tolerance of DTX-NPs in mice was increased greatly, allowing a higher dose to be safely intravenously injected. In addition, at the same dose (7.5 mg/kg), the antitumor effects of DTX-NPs were higher than that of DTX injection, which might be due to the passive targeting ability of DTX-NPs to tumor tissue. Therefore, DTX-NPs fabricated by our self-assembly strategy showed higher promise in their safety and effectiveness than the traditional DTX injection.
Declaration of interest

The authors report no declarations of interest.

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