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Facile preparation of cabazitaxel-loaded nanoparticles directly lyophilized from dioxane

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ABSTRACT Cabazitaxel (CTX) is currently formulated for clinical use in neat liquid surfactant (at a 27:1 mass ratio of Tween-80:CTX). We show here that CTX and Pluronic F127 can be dissolved together in a mixed solvent system comprising water and 1,4-dioxane, two commonly used freeze-drying solvents. This enables the sterile filtration of the mixture, subsequent lyophilization, and aqueous reconstitution of drug-loaded micelles. The micellization properties of the solvent system enabled sterile filtration only at low or high dioxane concentrations. Lyophilizate morphology and reconstituted micelle properties depended on the cosolvent/solvent ratio and the ratio of F127 to CTX, enabling the tuning of the size of reconstituted nanoparticles. A F127-to-CTX mass ratio of 3:1 by the post hydration method using 60% dioxane yielded good batch-to-batch reproducibility and resulted in micelles that were stable for at least 3 h following aqueous reconstitution. Upon intravenous administration to mice, CTX circulation in blood was not dependent on the micelle size and comparable to that of the neat Tween-80 formulation. *In vivo* antitumor efficacy in mice bearing human MIA Paca-2 tumors was also found comparable to that of the Tween-80 formulation. Taken together, these results demonstrate the utility of a simple CTX formulation methodology to produce a lyophilized drug product with a high drug-to-excipient ratio.

Keywords: cabazitaxel, lyophilization, nanomedicine, poloxamer, drug delivery, chemotherapy

INTRODUCTION

Taxanes, a family of microtubule-stabilizing drugs, have become broadly adopted in cancer chemotherapy, including the treatment of breast, lung, ovarian and prostate cancers [1]. Due to their hydrophobicity, taxane solubilization strategies are required, and the first clinical formulation of paclitaxel (Taxol®) was dissolved with Cremphor EL (polyethoxylated castor oil) and ethanol, with a mass ratio of Cremphor EL to paclitaxel of about ~88. In a typical 3-h infusion administration, patients receive up to ~29 g of Cremphor EL, leading to potential hypersensitivity reactions, sensory neuropathy, and nonlinear pharmacokinetics [2–4]. The desire to minimize potential side effects caused by surfactant carriers has motivated searches for alternative delivery methods. Abraxane®, also known as albumin-bound paclitaxel, comprises ~130 nm paclitaxel colloidal particles derived from human serum albumin and is finally lyophilized [5]. Because of the absence of Cremphor EL, Abraxane® has an increased maximum tolerated dose (MTD) compared with Taxol®, as well as a short time infusion without required premedication [2]. Abraxane® has shown superior antitumor efficacy compared with Taxol® and also the surfactant-dissolved Taxotere® (which is a Tween 80 surfactant formulation of docetaxel, another taxane member) [6,7]. Genexol-PM® is also a successful paclitaxel formulation which has been approved for the treatment of breast cancer and lung cancer in Korea [8]. It uses a biodegradable amphiphilic block copolymer to form paclitaxel micelles and is lyophilized for storage [9]. Genexol-PM® exhibited an even higher MTD than Abraxane and linear pharmacokinetics with dose [10]. To avoid the formulation in liquid surfactant or organic solvents, Abraxane® and Genexol-PM® require lyophilization, since taxanes are prone to rapid aggregation in aqueous solution.

Cabazitaxel (CTX) is a second-generation taxane designed to overcome the multidrug resistance observed after docetaxel or paclitaxel treatments owing to low affinity to the P-glycoprotein [11,12]. Studies have shown that CTX can also inhibit androgen receptor (AR) functionality, and AR and AR-associated heat shock protein (HSP) expressions, which will disturb prostate cancer growth [4,13]. In a phase 3 clinical trial, CTX was shown to extend the overall survival of metastatic castration-resistant prostate cancer patients from 12.7 months with mitoxantrone treatment to 15.1 months [14]. Its Jevtana® formulation typically comprises of 40 mg of CTX dissolved in 1 mL Tween 80, which is further dissolved using a 13% ethanol dilutent just prior to administration [3,4]. In recent years, CTX has also been widely formulated in many novel drug delivery systems, including micelles [15–26], serum albumin [27–32], liposomes [33–37], lipid nanoparticles [38–43], polymeric nanoparticles [44–46] and covalent conjugates [47–54]. However, due to the hydrophobicity properties of CTX, and the aqueous instability issue of

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CTX formulation, it is common to dissolve them with surfactants or alternatively other suitable drug carriers for intravenous administration. However, drug vehicles may increase the risk of adverse effects, hypersensitivity reactions, altered pharmacokinetics profile, etc. [3,4,55–58]. Therefore, long-term storage stability and reduced excipient addition are preferred for CTX formulations.

Previously we demonstrated a "surfactant-stripping" approach for generating Pluronic F127 (Poloxamer 407) poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) micelles loaded with CTX as an aqueous suspension with high drug-to-excipient ratios after stripping excess surfactant [21–23]. To enhance aqueous colloidal stability, various hydrophobic "co-loader" molecules, including mifepristone and clotrimazole, were identified from a screen. However, these compounds are pharmologically active and therefore could induce their own side effects. We therefore sought to develop a lyophilized formulation of CTX with a high drug-to-excipient ratio, as lyophilization is also established for other taxane formulations, such as Abraxane and Genexol-PM [59]. Our approach was made possible by the finding that CTX and F127 are both soluble with high concentrations in a water/1,4-dioxane co-solvent system. After lyophilization, the resulting CTX cake with minimal surfactant could be reconstituted to generate injectable CTX nanoparticles. Organic/water co-solvent lyophilization systems can offer potential advantages of varied sublimation rate, reduced freeze-dry time, and enhanced physical properties of the lyophilizate cake [60].

EXPERIMENTAL SECTION

Materials

The materials suppliers and details are as follows: CTX (Carbosynth # FC19621), Pluronic F127 (Sigma # P2443), Pluronic F108 (Sigma # 542342), Kolliphor P188 or Pluronic F68 (Sigma # 15759), 1,4-dioxane (Avantor # 4937-04), phosphate buffer saline (PBS, Gibco # 14190-144), Brij 97 (Spectrum # B1679), Cremophor EL (Sigma # C5135), Span 80 (TCI # S0060), Tween 80 (Sigma # P1754), Kolliphor HS 15 (Sigma # 42966).

Preparation of lyophilized CTX

Pre-lyophilized CTX solution was prepared by dissolving CTX and F127 or other surfactants in 1,4-dioxane, then adding 2 mmol L^{-1} PBS and mixing well. Briefly, for 5% dioxane formulations, CTX and F127 (or other surfactants) were dissolved in 50 μL of dioxane, and 950 μL PBS (2 mmol L^{-1}) was added. Then the vials were inverted 10 times until the solution was mixed well. For 60% dioxane formulations, CTX and F127 (or other surfactants) were dissolved in 600 μL of dioxane and added into 400 μL PBS (2 mmol L^{-1}), and the vials were inverted 10 times till the solution was mixed well. Pre-lyophilized CTX solutions were filtered by sterile nylon filter (0.22 μm Celltreat Lot: 190611-051 or 0.45 μm Celltreat Lot: 181011-051) before lyophilization if needed. To lyophilize the samples, they were frozen to −80°C for at least 6 h and the lyophilization process in the freeze-dry machine (Labconco Catalog #700401000) lasted at least 24 h. The resulting powders were sealed in vials wrapped with parafilm until reconstitution with PBS. To rehydrate the lyophilized powder, PBS was added, followed by standing for 3 min. The vials were tilted or inverted so that all powder was submerged in solution after standing for 5 min.

SEM and TEM

Scanning electron microscopy (SEM) of these samples was performed using a Carl Zeiss AURIGA CrossBeam Electron Microscope. All samples were fixed on a conductive carbon tape and mounted on the support and then sputtered with an approximately 2-nm layer of gold (Au) for 30 s with a sputter-coating unit (SPI-Module Sputter Coater). The morphology of hydrated lyophilized CTX micelles was observed by using a transmission electron microscope (TEM, JEM-F1400, JEOL) with negative staining by 1% uranyl acetate.

HPLC quantification

The CTX concentration in the solution was quantified by high-performance liquid chromatography (HPLC) analysis (Water Alliance 2790 HPLC with a C8 column). Briefly, 10 μL of sample stored in the indicated conditions was added into 190 μL dimethylsulfoxide (DMSO), vortexed until dissolved, and centrifuged for 3 min at 10,000 ×g. The resulting supernatant was transferred to HPLC vials and subjected to HPLC analysis. The elution gradient was linear from 20% to 70% acetonitrile in 0.1% trifluoroacetic acid at room temperature. The CTX concentration in the solution was measured by integrating the peak at a wavelength of 230 nm and comparing with a standard curve of pure CTX.

Characterization of lyophilized CTX

Nanoparticle sizes were measured with dynamic light scattering using a NanoBrook 90 Plus PALS instrument after 100-fold dilution in deionized water. The solution turbidity was measured by the absorbance at 600 nm *via* a Lambda XLS spectrophotometer (PerkinElmer).

Pharmacokinetics profile

The fresh hydrated lyophilized CTX formulation was quantified by HPLC analysis prior to the injection. CTX formulations (10 mg kg^{-1}) were bolus administrated to mice intravenously *via* the tail vein. At certain time points, 20 μL blood was collected from the ophthalmic vein. The blood samples were centrifuged at 2000 r min^{-1} for 10 min. The serum was collected and 10 μL internal standard (d6-CTX) was added, mixed with vortex, and stored at −20°C until HPLC analysis. To quantify the CTX concentration, 300 μL tert-butyl methyl ether was added to serum, vortexed and sonicated till mixed. The samples were centrifuged at 10,000 r min^{-1} for 3 min, and the tert-butyl methyl ether supernatant was collected. The extraction was repeated twice and purged with nitrogen until dry. The dried sample was reconstituted with 150 μL of 50% acetonitrile/water with vortex until fully dissolved.

The CTX concentration was determined by liquid chromatography/mass spectrometry (LC/MS) analysis. The samples were transferred to HPLC vials with insert. The LC/MS was performed using a Sciex API 3000 triple quadrupole mass spectrometer equipped with a Turboionspray source and a Shimadzu Prominence HPLC system. The HPLC system included two LC-20AD pumps, an online DGU-20A5R degasser, a CTO-20AC column oven and an SIL-20AC autosampler. The analytical column was a Waters 2.1 mm × 100 mm XSelect CSH C18 column (particle size 3.5 μm). The injection volume was 10 μL, and the needle wash was 50/50 and 70/30 acetonitrile/water. The LC flow rate was 200 μL min^{-1}. The mobile phases consisted of (A) 5/95 acetonitrile/water + 0.1% formic acid, and (B) 95/5

acetonitrile/water + 0.1% formic acid. The starting mobile phase was 60% B and was increased to 95% B over 5 min, and it was held at 95% for 3 min before equilibrating for 5 min. Multiple reaction monitoring (MRM) conditions for the CTX, including *m*/*z* of MRM pairs, collision energy, and orifice potential, were optimized by flow injection analysis. The MRM transitions for CTX and the deuterated internal standard (d6-CTX) were 836.7/555.5 and 842.5/561.4, respectively. For the LC/MS, the dwell time of each MRM transition was 300 ms, and the pause time for scan parameter changes was 5 ms. The CTX quantification limits were 2.5 ng mL^{-1}. Pharmacokinetic parameters were evaluated with PKSolver in Excel software.

Cell cytotoxicity assay

Mia Paca-2 cells were cultured in Dulbecco's modified eagle medium (DMEM) medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin. Cells were first cultured in 25 cm^2 flasks maintained at 37°C and 5% CO_2, and then seeded at a density of 10^4 cells/well and allowed to adhere for 24 h. To perform the cell cytotoxicity test, the cell medium was removed and the cells were washed with PBS twice. The CTX formulations were mixed into the medium and added to the wells. After the cells were incubated for 48 or 72 h, the medium was removed and the cells were washed twice with PBS. CCK-8 solution was added and cultured for 4 h, and the absorbance at 450 nm was measured. Cell viability was calculated as a ratio of viability of treated cells to untreated cells. All experiments were carried out at least in triplicate wells. As for the observation of live and dead cells, the cells were washed and stained with calcein AM and propidium (PI) according to the manufacturer's instructions, and then the images were recorded with an optical microscope (Nikon, Japan).

***In vitro* hemolysis assay**

Fresh red blood cells (RBCs) were obtained from mice and suspended in citrate; the erythrocyte suspension was obtained by centrifugation at 240 ×g for 13 min with brake setting 0, and then washed with PBS solution three times, followed by centrifugation at 1200 ×g for 5 min with brake setting 2. PBS solution was added and mixed to form an erythrocyte suspension in the original whole blood volume. Erythrocyte (15 μL) was mixed with 5 μL of CTX formulations at different concentrations and incubated at 37°C for 1 h. PBS (1 mL) was added and centrifuged at 3000 ×g for 5 min. PBS and dilute Triton X-100 solution were used as negative (0% lysis) and positive (100% lysis) controls, respectively. The absorbance of the supernatant was measured at 540 nm. The lysis cells were observed by an optical microscope (Nikon, Japan).

Tumor growth inhibition

The animal studies were performed in compliance with the protocols of Institutional Animal Care and Use Committee of the State University of New York at Buffalo. Female athymic nude mice were inoculated near the right groin with 5×10^6 Mia Paca-2 cells. When tumor sizes reached around 100 mm^3, the mice were randomly divided into three groups. The mice bearing tumors were untreated or treated by intravenous administration at the tail vein with a drug dose of 20 mg kg^{-1} CTX on day 1 and day 5. The tumors were monitored and the mice were sacrificed when tumor volumes exceeded 10 times the initial volumes or at the end of the study period.

Toxicity studies

Healthy female CD-1 mice were randomly divided into three groups (n = 5): PBS, lyophilized CTX and Tween 80-CTX. On day 0, 30 mg kg^{-1} CTX (a single dose) was intravenously injected into each mouse *via* the tail vein for each group. Mouse weights were monitored for 14 days. All mice were sacrificed on day 14, and then blood was collected *via* face piercing, suspended in citrate and kept for further complete blood count (CBC) analysis. Organs including hearts, livers, spleens, lungs, kidneys were harvested and fixed in formalin solution for further hematoxylin and eosin (H&E) staining analysis.

RESULTS AND DISCUSSION

Fig. 1a schematically illustrates the lyophilization process of CTX. Due to the limited solubility of CTX in water, 1,4-dioxane (freezing temperature of 11°C) was assessed as a freeze-drying solvent. The more commonly used freeze-drying solvent tert-butyl alcohol (TBA) was not effective in dissolving CTX, whereas dioxane was effective over the entire range of CTX concentrations assessed (Fig. 1b). F127 was highly soluble in both dioxane and water. As shown in Fig. 1c, when the dioxane volume in water was 5%, CTX with F127 (1 mg CTX and 3 mg F127 dissolved in 50 μL dioxane and 950 μL PBS) could effectively pass through membrane filters at recovery rates of 95% and 90% with filter pore sizes of 0.45 and 0.22 μm, respectively. Sterile filtration using a 0.22-μm filter is generally used for ensuring parenterally administered pharmaceuticals free from bacterial contamination. At either below or above 5% dioxane, less CTX passed through these filters. We hypothesize that a concentration of 5% dioxane assist in solubilizing the drug. As demonstrated by Bodratti *et al.* [61], the F127 surfactant is prone to form micelles at around 0.7 w/v%. But the F127 concentration in 5% dioxane with water solution was 0.3 w/v%. It is possible that PBS affected the micelle formation. Above 5% dioxane, the co-solvent system likely prevented micelles from effectively forming by reducing the free energy penalty for CTX to be encapsulated in the micelle hydrophobic core. Interestingly, when the proportion of dioxane is increased further and exceeds 50%, CTX passes through the sterile filters completely, as at that point CTX is likely to form a conventional solution.

Considering the drug recovery rate (95% and 90%) of sterile filtration at either 5% or 60% dioxane, these two concentrations were selected for the following lyophilization studies. First, we assessed whether F127 was advantageous for forming CTX micelles compared with other surfactants, a phenomenon we previously demonstrated [23]. As shown in Fig. 1d, F127 renders the best CTX solubility upon hydration among all the surfactants evaluated including Tween 80, HS15, P188, Crem EL, F68, Span, and Brij. Poloxamer (PEO-PPO-PEO) amphiphilic triblock copolymer surfactants such as F127 have been approved by Food and Drug Administration (FDA) as food additives and pharmaceutical ingredients [62–64]. The higher loading efficiency of F127 with CTX may be due to the fact that F127 has long hydrophilic PPO blocks that can form substantial hydrophobic domains in the micelle and the high-molecular-weight drug can be carried in bigger micelles [65].

Due to the different sublimation rates of dioxane and water, the appearance of the lyophilization cakes produced by freeze-drying varied, as shown in Fig. 2a. The lyophilized cakes produced by 5% dioxane were mostly collapsed, whereas the cakes produced by 60% dioxane were well-shaped and homogenous.

The phenomena may result from the higher freezing temperature of dioxane that gave rise to more stable cakes during the freezing process. The poor-quality cakes lyophililzed with 5% dioxane could be improved with an increasing proportion of F127, so that the obtained cakes became firm, especially after the mass ratio of F127-to-CTX reached 9, where the cakes with 5% and 60% dioxane were almost the same. The changes in morphology may be due to F127 occupying space and providing bulk to the lyophilization cake. As shown in Fig. 2b, c, the difference between 5% and 60% dioxane in the lyophilization solvent is apparent from the SEM images. When the water component of lyophilized sample is high (5% dioxane), the cake was dense and easily collapsed. When the proportion of the lyophilizing solvent dioxane is high (60%), the gap between the cakes is larger, making the cakes more elegant. This morphology difference might result from the different sublimation rates of dioxane and water.

The stability of lyophilized CTX upon hydration with PBS was investigated in a 24-h period. To rehydrate the lyophilized CTX powder, PBS was gently added to the vials which was then left standing for 3 min. Powder was submerged by the solution, and if not, the vial was tilted or inverted to ensure that the solution touched all floating powder. The hydration process took less than 5 min. The size of hydrated CTX cakes formed from 60% dioxane without sterile filtration of the solvent (Fig. 3a) is similar to that of the filtered groups (Fig. 3b). By increasing F127-to-CTX ratio from 2.5 to 9, nanoparticle sizes decreased from ~400 to ~100 nm when the ratio reached 7 and 8 in the groups with and without prefiltering, respectively. Turbidity measurements also confirmed the trend of size changes in these groups (Fig. 3c). The filtered group with 60% dioxane showed smaller size variation than pre-filtered groups. For the 5% dioxane groups, since CTX and F127 might form micelles upon being mixed before lyophilization, the size became bigger than that of 60% dioxane groups (Fig. 3d). But upon filtration by 0.22-μm nylon filters, the size became smaller (Fig. 3e). The size

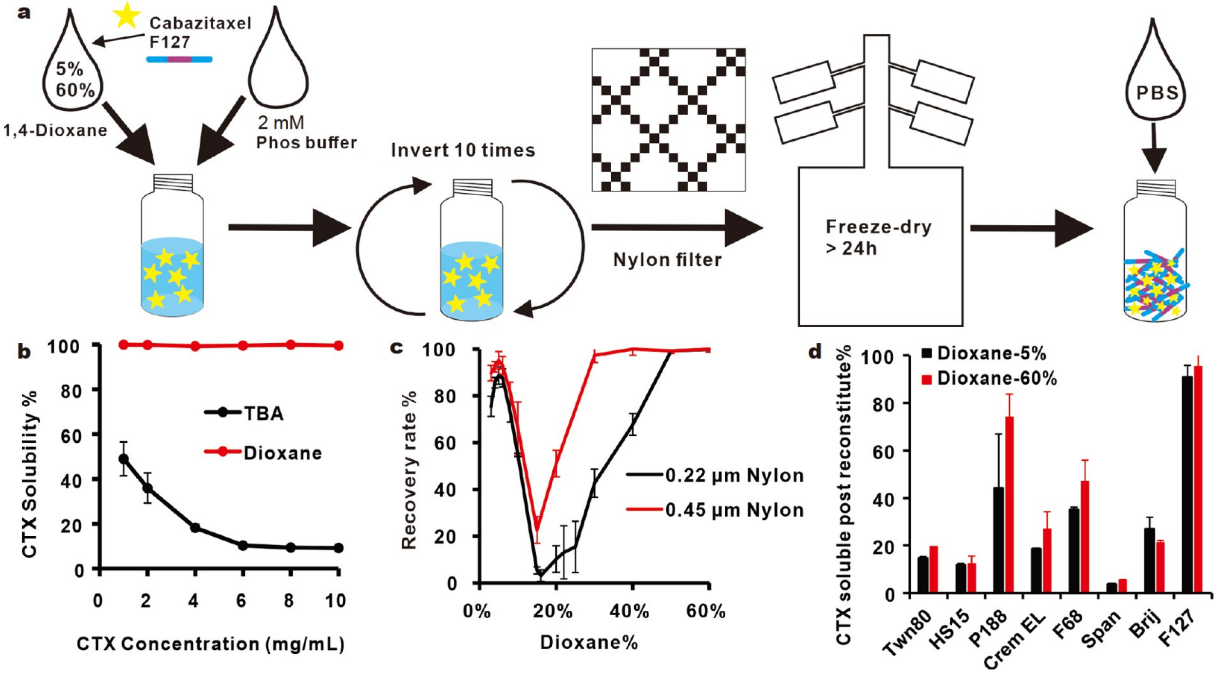

Figure 1 Lyophilization process of CTX with F127. (a) Schematic illustration of the CTX lyophilization process. (b) Solubility of CTX in TBA and 1,4-dioxane. (c) Effect of the dioxane percentage on the recovery rate of pre-lyophilized CTX solution after passing through sterile nylon filters with 0.22 and 0.45 μm pores, respectively. (d) Drug solubility tests of different surfactants by directly hydrating lyophilization samples. Data are shown as mean ± standard deviation (SD) for n = 3.

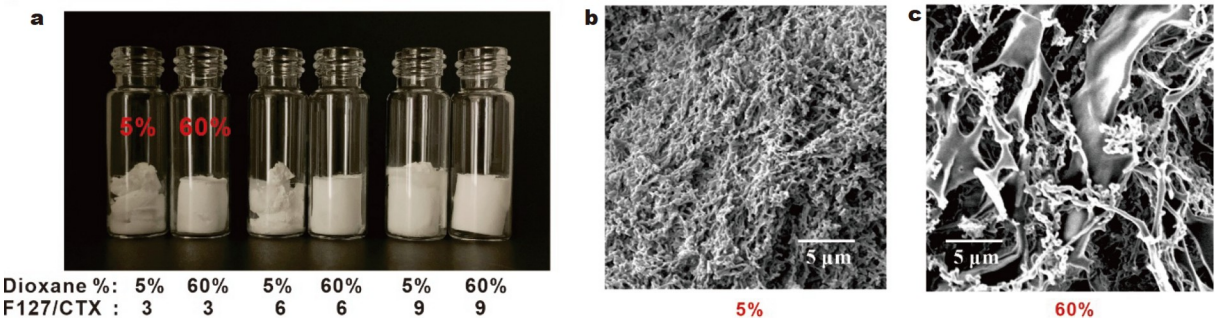

Figure 2 Appearance of the lyophilized cakes with different proportions of dioxane and different F127/CTX ratios. (a) Appearance of freeze-dried cakes with different ratios of F127/CTX. SEM images of freeze-dried cakes with F127/CTX ratio at 3: (b) 5% dioxane, (c) 60% dioxane.

decreased and stabilized around 100 nm when the ratio of F127/CTX reached 6 and 7 for the prefilter groups and groups without prefilter, respectively (Fig. 3e). The turbidity data also verified the trend of size change in these groups (Fig. 3f).

Since the sizes of the lyophilized CTX with 60% and 5% dioxane have no significant difference, and the sample of 60% dioxane can pass the filter more easily according to Fig. 1c, resulting in less CTX drug loss during preparation, the 60% dioxane condition with a 0.22-μm nylon filter was selected in the following study. The clinical CTX formulation Jevtana® with a surfactant to drug mass ratio of 26.5 and F127/CTX ratio of 3 was chosen for comparison, since it has nearly ten times less surfactant added [3]. With six independent replicates and six individual vials for each replicate, Fig. 4a shows that the lyophilized CTX has good reproducibility at F127/CTX ratio of 3. After hydration, the sample size is about 400 nm, and the variation range is between 300 and 500 nm. In the stability test, the particle size of the sample was well maintained with a negligible change within 3 h, providing sufficient time for clinical infusion administration. TEM of hydrated lyophilized CTX with a F127/CTX ratio of 3 confirmed that the spherical shape was well preserved (Fig. 4b).

The *in vitro* cytotoxicity of PBS-hydrated lyophilized CTX prepared with 60% dioxane and a 0.22-μm nylon filter was compared with free CTX dissolved in DMSO using a standard CCK8 cell viability assay in MIA Paca-2 human pancreatic tumor cells [37]. After incubation with CTX formulations for 48 h, cell death increased with increasing CTX doses (Fig. 5a). However, between 10 and 500 μg mL^{-1}, freeze-dried CTX was found to be slightly less effective than Tween 80-based CTX. The reason may be that the reconstituted CTX micelles are large in size and have limited interaction with the cells, whereas free CTX can easily penetrate into the cells. However, after incubation for 72 h, the cytotoxicity of lyophilized CTX and Tween 80-based CTX became almost similar, likely due to the release of CTX from the micelles (Fig. 5b). The viability of MIA Paca-2

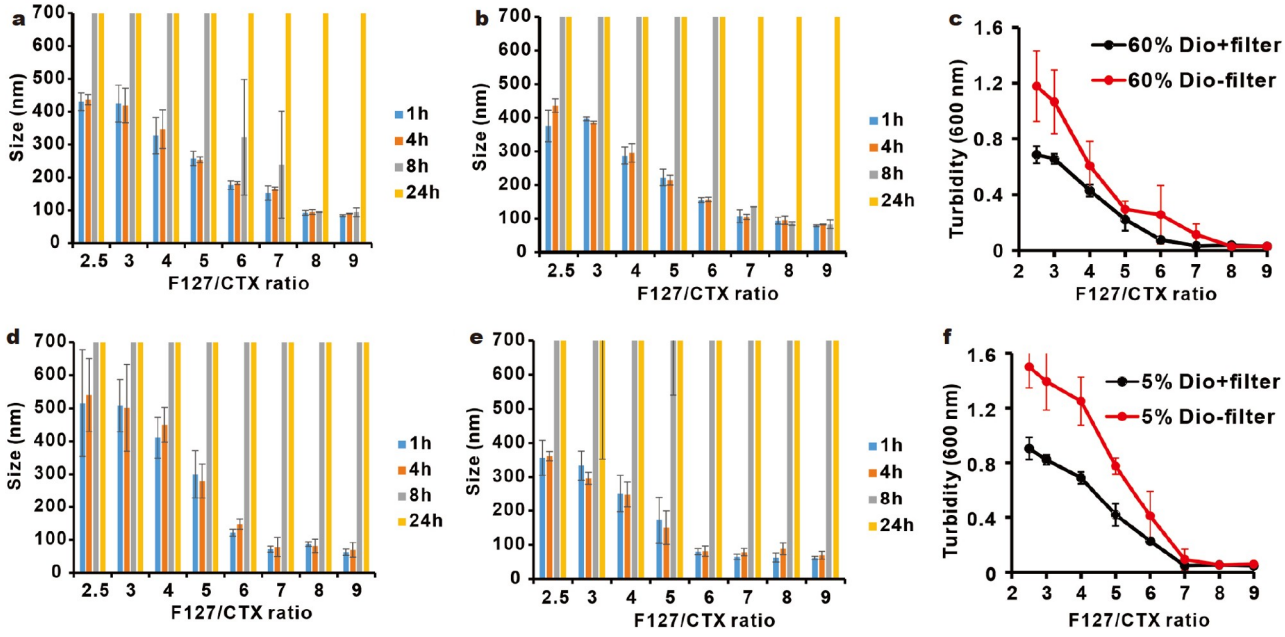

Figure 3 CTX lyophilization hydration trend with increasing amount of F127 added. The size stability of the lyophilized CTX prepared after hydration with PBS buffer by 60% dioxane with increasing F127/CTX ratio: (a) without prefilter, (b) prefiltered by a 0.22-μm nylon filter. (c) Turbidity (sample absorbance at 600 nm *via* ultraviolet-visible (UV-vis)) spectroscopy. The stability of the lyophilized CTX prepared by 5% dioxane with increasing F127/CTX ratio: (d) without prefilter, (e) prefiltered by a 0.22-μm nylon filter. (f) Turbidity (sample absorbance at 600 nm *via* UV-vis spectroscopy). Data are shown as mean ± SD for n = 3. Dio: dioxane; +/−filter mean the solution with or without filtration by a 0.22-μm nylon filter.

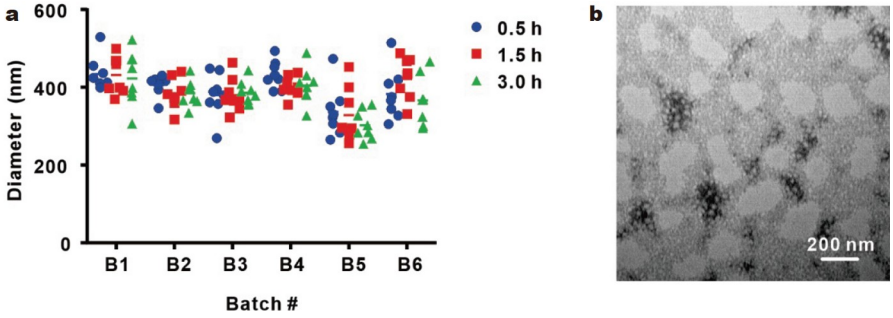

Figure 4 Characterization of lyophilized CTX at the F127/CTX ratio of 3. (a) Reproducibility of lyophilized CTX. With six independent replicates and six individual vials for each replicate (F127/CTX ratio of 3 with 60% dioxane), the size stability was recorded after hydration for 3 h. The one-way analysis of variance (ANOVA) *via* the Tukey method indicates there is no statistics significance between these groups. (b) TEM image of rehydrated lyophilized CTX.

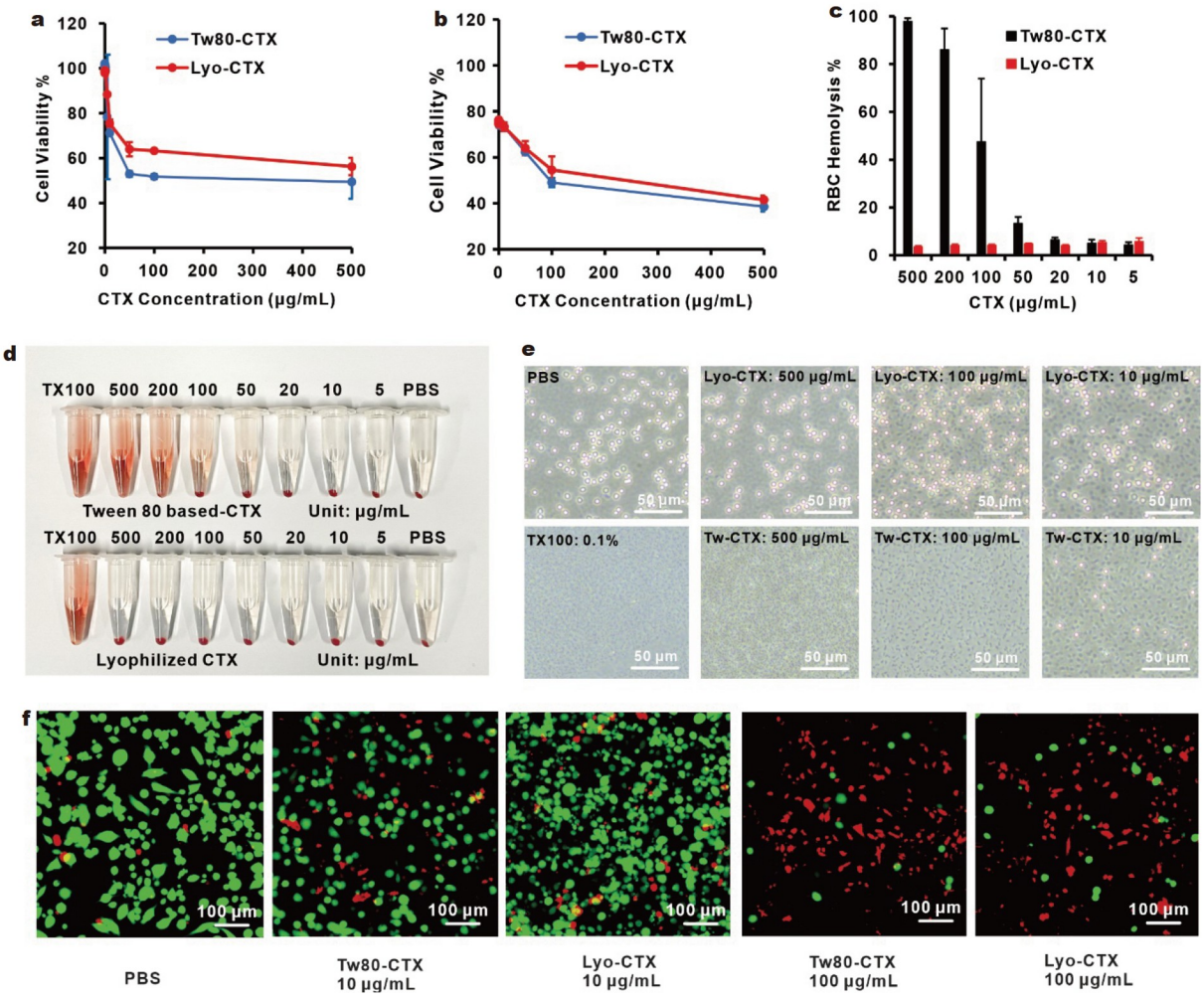

Figure 5 *In vitro* study of CTX formulations. Cell viability of lyophilized CTX and free drug on MIA PaCa-2 cells after incubation for (a) 48 and (b) 72 h, using CCK8 cell viability assay. MIA PaCa-2 cells were treated with free CTX or lyophilized CTX formulations (CTX concentration ranging from 0.1 to 500 μg mL^{-1}). The cells were incubated for 48 and 72 h post treatment. Data are shown as mean ± SD for $n = 5$ separately prepared wells. (c) Hemolysis assay of RBCs after incubation with lyophilized CTX or Tween 80-based CTX formulations. (d) Photograph of hemolysis of RBCs treated with lyophilized CTX or Tween 80-based CTX formulations. (e) Optical images of RBCs treated with lyophilized CTX or Tween 80-based CTX formulations in the hemolytic study. (f) Live/dead staining after cells were incubated with different formulations for 72 h.

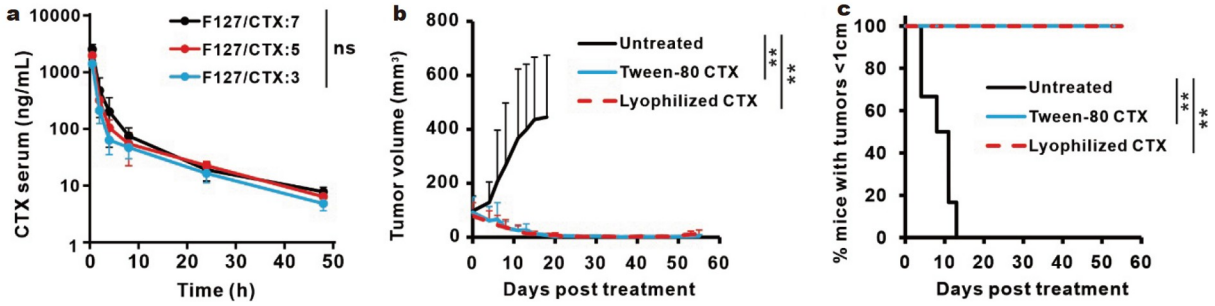

Figure 6 *In vivo* characterization of CTX formulations. (a) Serum pharmacokinetics profiles of different CTX formulations as indicated after intravenous bolus injection of lyophilized CTX at 10 mg kg^{-1} dose in BALB/c mice. The one-way ANOVA analysis (Prism GraphPad 6) indicates there are no statistically significant differences between these groups. (b) Tumor growth curves in inhibition study on Mia Paca-2 tumor-bearing nude mice. The mice were untreated or intravenously injected with lyophilized CTX or Tween-80-based CTX on day 1 and day 5 with a 20 mg kg^{-1} CTX dose. On day 18, the tumor volume of the untreated group had a significant difference compared with those of Tween-80-based CTX group and lyophilized CTX group, analyzed by one-way ANOVA statistics in Prism 6. (c) Percentage of mice with tumors less than 1 cm. Each data point shows mean ± SD for $n = 6$. $^{**}p < 0.01$, analyzed by log-rank test statistics in Prism 6.

human pancreatic tumor cells treated by these formulations after 72 h was also evaluated by live/dead staining analysis (Fig. 5f). At 10 μg mL^{-1} CTX dose, lyophilized CTX has a similar cell survival rate compared with the Tween 80-based CTX group.

Red fluorescence was observed when the CTX dose increased to 100 μg mL^{-1}, indicating the death of MIA Paca-2 cells. In contrast, for the PBS group, we only observed spotty cells with red fluorescence. A hemolysis study was carried out to compare the disruption effect of different formulations on RBCs. Freshly collected mice erythrocytes were incubated with various formulations at 37°C, and PBS and Triton X-100 were used as negative control and positive control, respectively. As shown in Fig. 5c, d, hydrated lyophilized CTX formulations induced negligible hemolysis for all concentrations investigated, whereas Tween 80-based CTX formulation caused significant hemolysis with increased CTX concentrations. The microscopic image also confirmed the disruption of RBCs upon incubation with Tween 80-based CTX formulations with high concentrations (Fig. 5e). These results indicate the safety of lyophilized CTX formulations compared with Tween 80-based CTX formulation.

The serum circulation profile of PBS-hydrated lyophilized CTX prepared with 60% dioxane and a 0.22-μm nylon filter was assessed by intravenously administrating the formulations at 10 mg kg^{-1} (Fig. 6a). F127/CTX ratios at 3, 5 and 7 were chosen,

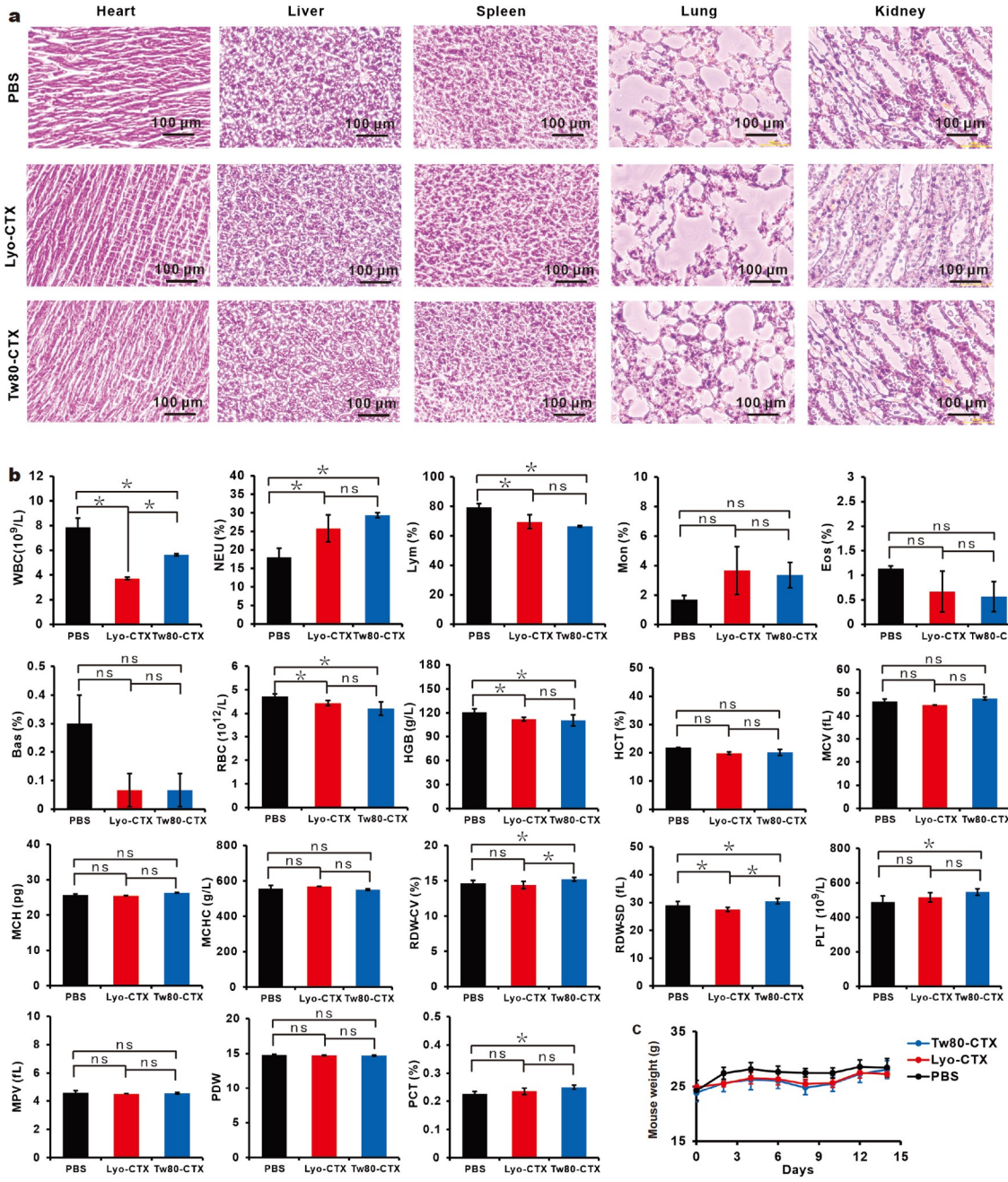

Figure 7 Toxicity of lyophilized CTX and Tween 80-based CTX formulations. CD-1 mice were received a single intravenous administration of 30 mg kg^{-1} CTX dose *via* tail vein on day 0 and sacrificed on day 14 for toxicity analysis (n = 5). (a) H&E staining analysis of major organs. (b) CBC analysis. Data were analyzed *via* t-test analysis, *p < 0.05. (c) Mouse weights after the mice were intravenously injected with 30 mg kg^{-1} CTX on day 0 and sacrificed on day 14 (n = 5). Standard range of mouse CBC: WBC (10^9/L): 0.8–6.8, NEU%: 15–45, Lym%: 55.8–90.6, Mon%: 1.8–6.0, Eos%: 1–6, Bas%: 0–1, RBC (10^{12}/L): 6.36–9.42, HGB (g/L): 110–143, HCT%: 34.6–44.6, MCV (fL): 48.2–58.3, MCH (pg): 30.2–35.3, MCHC (g/L): 320–360, RDW-CV%: 13–17, PLT (10^9/L): 450–1590, MPV (fL): 3.8–6, PCT%: 0.11–0.28, PDW%: 9.0–17.0.

since their sizes after hydration were approximately 400, 200 and 100 nm, respectively, representing a series of nanoparticle sizes to blood interaction. Based on non-compartmental pharmacokinetics analysis, the CTX half-life of lyophilized CTX at F127/CTX ratio of 3 was 11.3 h, which was slightly shorter than those for F127/CTX ratios at 5 (12.9 h) and 7 (12.6 h), although these differences were not statistically significant. As for the drug exposure to the blood assessment, lyophilized CTX formulations exhibit areas under curve (AUC) of 3647, 4823, 6306 ng mL^{-1} h for the F127/CTX ratios of 3, 5, and 7, respectively. It indicates that the formulation with smaller size was potentially circulated for a longer time owing to the enhanced permeation retention (EPR) effect. These formulations exhibited slightly longer half-lives than the Tween 80 CTX formulation, which we recently found to be 9.35 h when assessed in the same manner [21].

Next, the *in vivo* antitumor efficacy of lyophilized CTX with a F127/CTX ratio of 3 was evaluated in mice bearing Mia Paca-2 tumors. After tumors reached ~100 mm^3, the mice were received a 20 mg kg^{-1} CTX dose of lyophilized CTX or Tween-80-based CTX on day 1 and day 5, the tumor volume and mouse weight were monitored. As Fig. 6b shows, the tumors in the Tween-80 group and the lyophilized CTX group were shrunk significantly compared with the untreated group. The Tween-80-based CTX group exhibited a similar therapeutic effect as the lyophilized CTX group. At day 36, tumors were eradicated completely for three of six mice both in lyophilized CTX group and Tween-80-based group. The effective tumor therapeutic results were also confirmed with the percentage of mice with tumors less than 1 cm statistics (Fig. 6c). On day 11, all the mice in the untreated group have reached an endpoint, whereas the mice in the CTX treatment group showed 100% survival. The therapeutic efficacy of the Tween-80-based group and lyophilized CTX group had a significant ($p < 0.01$) difference compared with the untreated group under a log rank test.

After demonstrating the therapeutic efficacy, we next evaluated the toxicity of the lyophilized CTX formulation in CD-1 mice (6–8 weeks old). The mice were randomly divided into three groups. PBS, rehydrated lyophilized CTX (F127/CTX = 3, filtered through 0.22-μm pores) and Tween 80-based CTX were intravenously administered *via* tail vein at a single 30 mg kg^{-1} CTX dose on day 0. After 14 days, blood and organs were collected for CBC analysis and histological H&E analysis. As shown in Fig. 7a, major organs of the mice treated with either lyophilized CTX or Tween 80-based CTX exhibited no inflammation or other abnormality by H&E staining analysis, suggesting that lyophilized CTX did not cause acute toxicity. In addition, CBC analysis revealed that the administration of both lyophilized CTX and Tween 80-based CTX induced a decrease in white blood cells (WBC), lymphocytes (Lym), RBC counts, RDW-SD (red cell distribution width standard deviation) and hemoglobin (HGB) (Fig. 7b). These blood variations may be ascribed to the toxicity of CTX drug itself [11,21,66]. But other parameters including monocytes (Mon), eosinophils (Eos), basophils (Bas), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), mean platelet volume (MPV) and platelet distribution width (PDW) remained almost the same levels as the PBS group. Moreover, the Tween 80-based formulation, but not the lyophilized CTX, significantly affected RDW-CV (red cell distribution width coefficient of variation), RDW-SD, platelet (PLT) and platelet count (PCT) compared with healthy mice, which suggests that Tween 80 altered blood cell activity in blood circulation and induced more toxicity than the lyophilized formulation [3,67]. In addition, there was no difference of mouse weight after treatment with lyophilized CTX or PBS (Fig. 7c).

CONCLUSION

A lyophilized CTX formulation was produced that employed freeze-drying of mixed solvents to readily generate a lyophilized formulation of CTX and F127. Only a short time (<5 min) was required to hydrate the lyophilized CTX cakes using PBS and the rehydrated formulation was stable for at least three hours, which provides potential for infusion chemotherapy. Similar to Tween-80-based CTX formulation, lyophilized CTX has strong tumor inhibitory activity and can completely eradicate established human tumors. In summary, lyophilized CTX can be easily prepared and can substitute for Tween-80-based CTX formulations, but with a substantially larger drug-to-surfactant ratio.

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Conflict of interest The authors declare that they have no conflict of interest.

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一种用二氧六环制备紫杉醇冻干制剂的简易方法

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摘要 第二代紫杉醇卡巴他赛(CTX)由于对P-gp糖蛋白的亲和力较低, 具有不易产生耐药性等优点, 被成功地应用于治疗耐药性前列腺癌的临床治疗中. 由于CTX的疏水性较高, 临床使用的制剂是由液体表面活性剂Tween-80和乙醇组成的(Tween-80与CTX的质量比为27:1). 而Tween-80可能在血液中导致溶血问题, 引起过敏反应. 在此工作中, 我们报道了一种只用少量表面活性剂(pluronic F127与CTX的质量比为3:1)的冻干CTX的制剂: CTX和Pluronic F127一起溶解在由水和1,4-二恶烷(两种常用的冷冻干燥溶剂)组成的混合溶剂系统中制成的冻干粉. 胶束状态下的溶剂系统可以经过无菌过滤膜达到除菌效果, 结果表明在5%和高于60%的1,4-二恶烷浓度下通过过滤才能达到理想的过滤效果. 冻干CTX制剂和重溶胶束的特性取决于共溶剂/溶剂比和F127与CTX的比例, 并且能够通过调整比例来调节纳米颗粒的尺寸. 用60%的1,4-二恶烷溶解质量比为3:1的F127/CTX所得的冻干CTX产物, 重溶后的胶束在水相中能稳定至少三小时. 在对小鼠进行静脉给药后, 血液中的CTX浓度不依赖于胶束的粒径大小, 并且血液代谢和纯Tween-80制剂类似. 小鼠抗肿瘤实验表明小鼠体内MIA Paca-2 肿瘤抑制功效与Tween-80制剂相似. 综上所述, 此工作报道了一种简单的冻干方法用于制备紫杉醇药物制剂, 具有制备方法高效和表面活性剂添加量低等优点.

