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A surfactant-stripped cabazitaxel micelle formulation optimized with accelerated storage stability

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ABSTRACT

Pluronic (Poloxomer) micelles can solubilize cabazitaxel (CTX), a second generation taxane, and then be subjected to low-temperature "surfactant-stripping" to selectively remove loose and free surfactant, thereby increasing the drug-to-surfactant ratio. We previously found that addition of certain other co-loaded hydrophobic cargo to the micelles can result in stabilized, surfactant-stripped cabazitaxel (sss-CTX) micelles, which resist drug aggregation in aqueous storage, a common challenge for taxanes. Here, we show that elevated temperatures can accelerate the aggregation of sss-CTX micelles, thereby enabling rapid optimization of formulations with respect to the type and ratio of co-loader used for stabilization. A sss-CTX micelle formulation was developed using mifepristone as the co-loader, at a 60 % mass ratio to the CTX. Drug release, hemolysis and complement activation were investigated in vitro. Microtubule stabilization and in vitro cytotoxicity were similar for sss-CTX and a conventional Tween-80 micelle formulation. In vivo pharmacokinetics also revealed similar blood circulation of the two formulations. In subcutaneous Lewis lung carcinoma tumors, as well as in an aggressive mouse model of malignant pleural effusion, sss-CTX showed a similar therapeutic effect as the Tween-80 based formulation. Altogether, these data show that sss-CTX can achieve similar efficacy as conventional Tween-80 formulations, albeit with substantially higher drug-to-surfactant ratio and with capability of extended aqueous storage.

INTRODUCTION

There are approximately 10 million cancer deaths worldwide each year (1). Taxanes, namely paclitaxel, docetaxel, and cabazitaxel (CTX), are frequently-used chemotherapy drugs that stabilize microtubule dynamics of cancer cells, arresting cell mitosis, leading to cell death (2). CTX is a second generation taxane developed to overcome multi-drug resistance owing to its relatively low affinity to P-glycoprotein. In a phase III clinical trial, CTX treatment with prednisone extended patient survival compared to mitoxantrone in metastatic castration-resistance prostate cancer (3). The United States Food and Drug Administration approved the Tween-80 based CTX formulation Jevtana® for the treatment of metastatic castration-resistant prostate cancer in 2010.

As CTX is hydrophobic, solubilizing excipients or drug delivery systems must be used for intravenous administration. The Jevtana® formulation uses the liquid surfactant Tween-80 to dissolve CTX, with 40 mg per CTX in 1 mL of Tween-80. 13% ethanol is used for dilution prior to treatment. Aqueous nanoformulations of hydrophobic drugs have been extensively studied and offer the potential to modulate pharmacokinetics and biodistribution. Novel cabazitaxel drug delivery formulations have been reported with various characteristics, including serum albumin (4, 5), lipid particles (6-8), micelles (9-14) and covalent conjugates (15-17).

Previously, our lab introduced a so-called surfactant-stripping approach for delivery of hydrophobic drugs including CTX (18). Pluronic (Poloxomer) F127 triblock copolymer micelles feature a sensitive critical micelles temperature (CMT), so when the temperature decreases below the CMT, loose and unincorporated surfactant will shift from micelles to

unimers (19). By then removing unimers through a low-temperature filtration process, the solution can be concentrated without losing the active drug, leading to an increased ratio of drug to surfactant. However, this approach with CTX micelles resulted in a formulation with limited storage stability. We subsequently screened dozens of hydrophobic co-loaders to identify ones that can increase sss-CTX storage stability (20). In the present work, we show that elevated storage temperatures can provide a convenient means to rapidly optimize the stability of different CTX micelles formulations.

MATERIALS AND METHODS

Materials

The following materials were obtained: Cabazitaxel (Carbosynth Cat. # FC19621); deuterated cabazitaxel (1 mg reference standard; Toronto Research Chemicals Cat. # C046502-1MG); Pluronic F127 (Sigma Cat. # P2443); clotrimazole (Alfa Aesar Cat. # J63895), ketoconazole (Alfa Aesar Cat. # J63367), cobalt nitrate hexahydrate (Alfa Aesar Cat. # 36418), ammonium thiocyanate (Alfa Aesar Cat. # A10632), Tween-80 / Polysorbate 80 (VWR Cat. # EM-9490), nimodipine (TCI Cat. # N0896), and mifepristone (TCI Cat. # M1732). The following solvents were used: ethyl acetate (Fisher), acetone (Fisher), methylene chloride (Fisher), acetonitrile (Fisher), and ethanol (Decon).

Cabazitaxel and F127 quantification

To quantify the drug concentration in solution, 10 µL of sample stored in the indicated conditions was added into 190 µL DMSO, vortexed until dissolved, and centrifuged for 3 min

at 10000 g. The resulting supernatant was subjected to HPLC analysis (Water Alliance 2790 HPLC with a C8 column). The elution gradient was linear from 20 to 70% acetonitrile in 0.1% trifluoroacetic acid at room temperature. The CTX was measured by integrating the peak at a wavelength at 230 nm and comparing to a standard curve. The mifepristone co-loader was measured at a wavelength of 306 nm. To quantify F127, 100 µL of cobalt thiocyanate solution, 40 µL samples in the concentration range of 0-7.5 wt %, 200 µL ethyl acetate and 80 µL ethanol were mixed and vortexed for 10 seconds. The cobalt thiocyanate solution was prepared by dissolving 0.3 g cobalt nitrate hexahydrate and 1.2 g ammonium thiocyanate in 3 mL water. The mixed sample was vortexed for 10 s and centrifuged at 10000g for 3 min. The blue supernatant was removed and the blue pellet was washed with ethyl acetate several times until the washed supernatant had no color. The pellet was dissolved in 1 mL acetone and the absorbance at 623 nm was measured by a Lambda XLS spectrophotometer (PerkinElmer).

Formulation preparation and assessment

For optimization of sss-CTX preparations, 4 mg cabazitaxel and 2.4 mg co-loader were dissolved in 0.2 mL methylene chloride (DCM), then slowly added dropwise to 1 mL 10% F127 solution. The solution was stirred until clear (~5 hr). To carry out surfactant-stripping, the resulting 1 mL solution was diluted with 10 mL cold phosphate-buffered saline (PBS) in a centrifugal filtration unit (MWCO: 100,000 kDa) and centrifuged at 3000 g at 4 °C for 30 min. The filtrate was removed and the washing process was repeated two more times, each time with addition of 10 mL cold PBS.

For large-scale sss-CTX, 300 mg CTX and 180 mg mifepristone were dissolved in 20 mL

DCM, then added drop-wise into 100 mL 10% F127 solution, stirred for at least 5 hr to clear. The resulting clear solution was diluted with 500 mL cold PBS and subjected to diafiltration (Sartorius Vivaflow # 1501008VS, 100 kDa MWCO) at 4 ℃ to remove excessive F127 until 100 mL concentrate was left. To maintain the temperature, the entire membrane system and sample were placed in ice. Cold PBS solution was added back and the wash process was repeated 5 times. After each washing, 2 mL of filtrate was collected for drug and F127 quantification. The resulting washed solution was adjusted to 50 mL by adding PBS, then passed through 0.2 μm sterile filter. Formulations were stored in the indicated conditions and soluble drug content was assessed as described above.

For Tween-80 based CTX, 40 mg CTX was dissolved in 1 mL Tween-80, and sonicated and mixed until completely dissolved. Before use, 3 mL 13% (wt/wt) ethanol was added to the solution and dissolved by water bath sonication. The solution was diluted to desirable concentration by PBS.

Size and zeta potential measurement were carried out with dynamic light scattering using a NanoBrook 90Plus PALS instrument after 100-fold dilution in deionized water.

Electron microscopy was carried out with a JEM-2010 electron microscope with MIF-sss-CTX placed on a Formvar/Carbon Coated - Copper 200 mesh grid and negatively stained with 2% uranyl acetate.

In vitro drug release

The in vitro release of CTX and MIF from the free drug solution and MIF-sss-CTX nanoparticles was investigated using a dialysis membrane method. Dialysis bags (12–14 kDa

MWCO) were suspended in pH 7.4 phosphate-buffered saline containing 3% Tween 80 and maintained at 37 °C in a stirring water-bath at 100 rpm. At designated time intervals, samples of the release medium were withdrawn followed by compensation with the same volume of fresh release medium. All samples were run in triplicates and filtered through a 0.45 μm membrane filter. The amount of CTX and MIF released was analyzed by HPLC equipped with a C8 column as mentioned above. The cumulative amount of drug release over the time period was plotted against time.

Tubulin Inhibition

The kinetics of tubulin polymerization was performed by tubulin polymerization assay kit (BK006P, Cytoskeleton, Denver, Co, USA). The purified porcine brain tubulin was diluted with tubulin buffer to 3 mg/mL and frozen in -80 °C before use. To measure tubulin polymerization, the tubulin solution was mixed with 10% glycerol, 1 mM GTP and 10 μM drug formulations. The mixture was pre-heated to 37 °C and after addition was quickly transferred to a 96-well plate reader (TECAN Safire 2) to read the absorbance every minute for 30 min at 340 nm at 37 °C.

Blood interaction studies

Fresh human erythrocytes were collected in citrate from healthy adult human volunteers following informed consent under protocols approved by the University at Buffalo Health Science Institutional Review Board. The erythrocyte suspension was obtained by centrifugation at 300 g for 13 min with the brake setting 0. The supernatant plasma was

stored for further complement activation study use. The erythrocyte suspension was washed by PBS solution three times with 1200 g for 5 min with brake setting 2. PBS solution was added to form an erythrocyte suspension in the original whole blood volume. To evaluate hemolysis, 15 μL of erythrocyte was mixed with 5 μL of CTX formulations at different concentrations, then incubated at 37 ◦C for 30 min. 1 mL PBS was added and centrifuged 3,000 g for 5 minutes. PBS solution and diluted Triton X-100 solution were used as a negative (0% lysis) and positive controls (100% lysis), respectively. The absorbance of the supernatant was measured at 540 nm with a plate reader.

For measurement of complement activation, fresh human plasma was obtained by centrifuging whole blood as mentioned above. 15 μL of plasma was mixed with 5 μL CTX formulations at concentrations quantified by HPLC. After incubating the mixture at 37 °C for 30 min, the complement reaction was stopped with 980 μL specimen diluent added provided by the supplier. Scb5-9 amounts were quantified by ELISA kit (Quidel Cat. # A020) following manufacturer instructions.

Cell Viability Study

LLC cells (5000 cells/well) were seeded into a 96-well plate with 100 μL culture medium and allowed to grow for 24 h before treatment. Then, the medium was replaced by 100 μL fresh culture medium containing various concentrations of MIF-sss-CTX or Tween-80 CTX formulations. After incubation for 24 hr at 37 °C, the cell viability was evaluated by the CCK-8 assay.

Pharmacokinetics

10 mg/kg CTX formulations were bolus administrated to mice intravenously via tail vein. At certain time points, small blood volumes were collected from the ophthalmic vein. The blood samples were centrifuged at 2000 rpm for 10 min. The serum was collected and 10 µL internal standard (d6-cabazitaxel) was added, mixed with vortex and stored at -20 °C until HPLC analysis. To quantify CTX concentration, 300 µL tert-butyl methyl ether was added to 50µL of serum, vortexed and sonicated to mix. The samples were centrifuged at 10000 rpm for 3 min, 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 50% acetonitrile/water with vortex until fully dissolved. The CTX concentration was determined by 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 2 LC-20AD pumps, an online DGU- 20A5R degasser, a CTO-20AC column oven and a SIL-20AC autosampler. The analytical column was a Waters 2.1. 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. 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, it was held at 95% for 3 min before reequilibrating for 5 minutes. MRM (Multiple reaction monitoring) 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-cabazitaxel) were 836.7/555.5 and 842.5/561.4 respectively. 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 ion spray voltage, declustering potential, collision energy and source temperature were 5500V, 22, 15 and 400oC respectively. The CTX quantification limits are 2.5 ng/mL. Pharmacokinetic parameters were evaluated with PKSolver in Excel software.

Tumor growth inhibition

Murine Lewis lung carcinoma cell line (LLC) was purchased from American Tissue Culture Collection (ATCC). For the murine malignant pleural effusion (MPE) model, LLC cells stably expressing firefly luciferase (LLC-Luc) were established. All cells were cultured in Dulbecco' s modified Eagle' s medium (DMEM) with 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin and streptomycin) at 37 °C with 5% CO2. Male C57BL/6 mice (6−8 weeks old) were purchased from Liaoning Changsheng Biotechnology Co., Ltd. All mice were raised under specific pathogen-free (SPF) condition in the Animal Center of Huazhong University of Science and Technology (HUST; Wuhan, China). All animal studies were approved by the Animal Care and Utilization Committee of Tongji Medical College, Huazhong University of Science and Technology. For mouse tumor model, mice were anesthetized using 1% pentobarbital sodium before all operations. LLC cells (8×10^{5} cells suspended in 50 μL PBS) were injected subcutaneously into the right flank region of each mouse. To establish the MPE model, mice were anesthetized with isoflurane before all procedures. LLC-Luc cells (2×10^{5} cells suspended in 50 μL PBS) were injected into the

pleural cavity through the right tenth or eleventh intercostal space at the midaxillary line. To ensure the successful establishment of MPE model, four days after inoculation of LLC-Luc cells, each mouse was observed by bioluminescence imaging. After the mouse tumor model and MPE model were successfully established, mice were randomly divided into three groups, including PBS (placebo control), the Tween-80 formulation (Jevtana®-like) and MIF-sss-CTX. For mouse tumor model, mice were treated by intraperitoneal injection on days 7, 9, 11 with 100 μL liquid (PBS, Tween-80 CTX or MIF-sss-CTX, 6 mg/kg). The tumor size was measured every other day using a vernier caliper. Tumor volume was calculated according to the formula: volume = $width^2 \times length/2$. Mice were sacrificed when the tumor volume reached 1,000 mm^3. For MPE model, mice were intrapleural injected with 50 μL liquid (PBS, Tween-80 CTX or MIF-sss-CTX, 8mg/kg) under isoflurane anesthesia on days 4, 6, 8. To evaluate MPE growth, 3 mice in each group were imaged on days 4, 12, 17 under 1% pentobarbital sodium anesthesia using the Bruker In Vivo MS FX PRO Imager. The remaining mice were observed until death. After MPE mice were anesthetized with 1% pentobarbital sodium solution, they were injected intraperitoneally with 150 mg/kg D-luciferin (Thermo Life, CAS: 103404-75-7). After 15 min, Bioluminescence was measured with a Spectral Instruments Imaging Optical Imaging Platform (Lago X, Cold Spring Biotech Corp.; 20 s exposure). Luminescent photographs were acquired with 3 min exposure time and X-ray images were taken with 30 s exposure time.

RESULTS AND DISCUSSION

Figure 1A schematically illustrates how the micelles strip excessive F127 under low temperature membrane filtering process to generate stabilized, surfactant-stripped cabazitaxel (sss-CTX). As surfactant-stripped CTX micelles are not stable in storage, to prolong nanoparticle stability, a stabilizing hydrophobic cargo molecule is co-loaded in the micelles that prevents taxane aggregation.

Figure 1B demonstrates how co-loaded cargo molecules in sss-CTX micelles can slow nanoparticle aggregation kinetics and different co-loaders lead to different performance. Surfactant-stripped cabazitaxel micelles on their own precipitated within 3 days. When sss-CTX was prepared with nimodipine (NIM; a hydrophobic calcium channel blocker) as the co-loader, CTX aggregation was delayed, with complete drug aggregation not occurring for a week of storage. However, clotrimazole (CLT; a hydrophobic antifungal agent) and mifepristone (MIF; a hydrophobic abortifacient) completely inhibited drug precipitation in sss-CTX micelles during the storage period. NIM, CLT and MIF were previously identified as CTX micelle stabilizers (20), although only CLT and MIF could lead to long term micelle stabilization.

Figure 1C directly compares the refrigerated storage stability of sss-CTX with the effective co-loaders (CLT and MIF) and the less effective one (NIM) with two storage durations. At 4 °C, drug aggregation kinetics were slower than at room temperature. Without any co-loader, all the CTX aggregated within a week of storage. However, NIM did not have any drug aggregation within a week, but did completely aggregate with a month of storage. On the other hand, CLT and MIF did not aggregate at all during refrigerated storage.

The results of **Figure 1** underscore some challenges of assessing the long-term stability of sss-CTX formulations under refrigerated storage conditions since precipitation can take many months to occur. This duration is not convenient for assessing stability and optimizing formulations. On the other hand, at room temperature, the aggregation of NIM-sss-CTX micelles was accelerated, compared to 4 °C. Based on this observation, we rationalized that sss-CTX formulations could be optimized for ideal co-loader concentration by simple storage at elevated temperatures. The specific use of Pluronic for accelerated temperature stability testing of sss-CTX is caveated given that the temperature sensitivity of the surfactant may cause specific micellization-related changes in the system behavior.

Figure 2A shows the aggregation kinetics of sss-CTX stabilized by another co-loader, ketoconazole (KTN), that we previously identified as a CTX micelle stabilizer, with intermediate efficacy, although not as effective as MIF or CLT (20). Increasing temperature during storage led to faster aggregation kinetics. No aggregation apparent with storage at 4 °C, but heavy aggregation occurring within a couple of weeks with room temperature storage, and within just days with storage at 37 °C. Therefore, using storage at 37 °C for accelerated stability testing, MIF and CLT were assessed to determine optimal sss-CTX composition. Different ratios of MIF or CLT were co-loaded along with CTX into surfactant-stripped micelles. Under these storage conditions, CLT exhibited marginal capacity to inhibit drug aggregation. MIF was more effective, and an optimal ratio of 60% MIF to CTX was found to promote the greatest soluble drug retention as in **Figure 2B**. We previously reported the mechanism of stabilization is likely through interaction with CTX in the hydrophobic micelles core, as CTX tends to self-aggregate (20). The MIF-sss-CTX

formulation with 60% co-loading was then evaluated for long-term storage stability at different temperatures (4, 25, 37, and 50 °C). As shown in **Figure 2C,** MIF-sss-CTX was stable for over 100 days without aggregation at 4 °C. Although we did not formally measure longer storage periods, we did observe that MIF-sss-CTX samples kept for over 600 days in refrigerated storage remained free of any noticeable precipitation. At 25 °C, MIF-sss-CTX remained stable with aggregation for 80 days. At 37 °C, aggregation occurred more rapidly, with the majority of the drug aggregated within a couple of months. At 50 °C, the CTX aggregated within days. The storage stability was compared to a Tween-80 formulation of CTX at the same concentration (5 mg/mL), that was diluted in a 13 % ethanol diluent, which is similar to the commercial CTX formulation. In storage, the diluted Tween-80 formulation could not remain stable without precipitation any longer than a few weeks at any storage temperature (**Figure 2D**). We also note that an additional saline dilution step is usually carried out for preparation of the clinical formulation for administration, which likely further increases the rate of drug precipitation. Indeed, after preparing Jevtana® for infusion, its administration is recommended within 8 hr of storage at room temperature, with the precaution noted to avoid use if there is any visible crystalized drug. Interestingly, at the highest temperature (50 °C), Tween-80 appeared to help the drug remain dissolved to some extent. Taken together, MIF-sss-CTX with a 60% co-loading ratio of MIF had excellent storage stability and was used for further studies.

A large-scale synthesis procedure using tangential flow filtration for surfactant-stripping was then carried out, rather than the small-scale approach that made use of micro-centrifugal filtration units. Filtration was carried out with ice bath cooling to maintain a temperature

below the CMT of F127. As shown in **Figure 3A**, CTX had an approximate 10 % loss after 5 washes while MIF had approximately 5% loss. Free F127 was nearly completely removed. The drug loss appeared to be due to leakage through the membrane used for tangential flow filtration. As shown in **Table 1**, after surfactant-stripping, MIF-sss-CTX has a drug to surfactant molar ratio of 4.65:1, which is approximately 80 times higher than that of the commercial Tween-80 based formulation. **Figure 3B** shows that the morphology of MIF-sss-CTX is small and generally spherical. Light scattering shows an average 40-50 nm hydrodynamic diameter with negative surface charge (Supplementary Figure 1). The release profile was assessed by dialyzing the CTX formulations against phosphate-buffered saline (PBS) containing 3% polysorbate 80 at 37 °C as a sink (**Figure 3C**). The result indicates that the free drugs of CTX or MIF in ethanol had fast release, and could completely permeate through dialysis membrane within 12 hr. However, the CTX and MIF in micelles only had a 15% release to the surrounding solution after 24 hr, exhibiting weak release from micelles, likely owing to the larger size of the micelles that are too large to pass through the membrane.

The mechanism of action of CTX is to interact with tubulin, leading to the stabilization of microtubules, cell arrest in mitosis, and ultimately apoptosis. Enhanced in vitro microtubule polymerization can be assessed directly to confirm taxane activity. Microtubule polymerization of MIF-sss-CTX was evaluated by a porcine tubulin assay, with all compounds initially assessed from DMSO stock solutions. CTX induced substantially faster tubulin polymerization than MIF, the co-loader used, which had polymerization consistent with a background polymerization level. The tubulin polymerization inhibitor vinblastine severely slowed tubulin polymerization. CTX appeared to induce similar or slightly more

polymerization compared to paclitaxel, a first generation taxane. As **Figure 4B** demonstrates, relative to the other samples, all aqueous CTX formulation induced similar tubulin polymerization stabilization rates. This implies that despite the difference in surfactant content and type, the micelles were sufficiently kinetically dynamic to enable rapid interaction of the CTX with tubulin.

Next, two in vitro blood interaction assays, hemolysis and complement activation were carried out with these formulations. It should be noted first that at the clinically used dilutions, hemolysis and complement activations are not problematic issues for Jevtana®. Hemolysis was assessed with fresh human erythrocytes to evaluate whether the red blood cells could be damaged by the formulation. Human erythrocytes were incubated with CTX at 37 ℃ for 30 min at the indicated concentrations. **Figure 5A** indicates that MIF-sss-CTX did not cause lysis of human red blood cells, while the Tween-80 based formulation did induce damage, specifically at higher CTX concentration. Even without surfactant-stripping, CTX dispersed in F127 micelles induced minimal hemolysis. This likely reflects the gentler nature of Pluronic compared to Tween-80 for dissolving membrane lipids. As **Figure 5B** shows, in vitro the Tween-80 based vehicle also induced a high level of activated SC5b-9 in human serum. In comparison, MIF-sss-CTX induced less complement activation level, which was likely due to the milder nature of F127, as CTX in F127 without stripping also had diminished complement activation.

Serum pharmacokinetics of MIF-sss-CTX and the Tween-80 CTX formulation were assessed in healthy mice and quantified by LC/MS with cabazitaxel-d as an internal standard. CTX was administered via tail vein injection at 10 mg/kg and serum was sampled at the time

points shown in **Figure 6.** Surfactant-stripped micelles and the Jevtana-like Tween-80 formulation showed similar behavior. MIF-sss-CTX had a serum half-life of 7.9 h and an AUC of 5881 ng/mL*h, while the Tween-80 based formulation exhibits a half-life of 7.7 h with an AUC of 5138 ng/mL*h. The similar serum pharmacokinetics of MIF-sss-CTX and the Tween-80 formulation might imply that once in the bloodstream, CTX rapidly reaches equilibrium and re-distributes from the micelles (surfactant-stripped or Tween-80) to circulating serum components such as albumin and lipoproteins. This is also generally consistent with the in vitro tubulin polymerization data, which showed negligible differences between the different solubilization approaches. Although surfactants such as Cremophor EL can alter the pharmacokinetics of taxanes (22), these data suggest that the administered Tween-80 in the vehicle at this dosage had minimal impact on circulation, compared to MIF-sss-CTX, which contained substantially less surfactant.

The *in vitro* cytotoxicity of the MIF-sss-CTX in murine LLC cells was next investigated. The tumor efficacy of MIF-sss-CTX and the Tween 80 based CTX formulation against LLC cells was evaluated by a CCK-8 assay. Similar as previous results for tubulin binding and pharmacokinetic behavior, MIF-sss-CTX showed a similar cell killing effect to the Tween-80 CTX based formulation (**Figure 7A**). Although cell uptake of the micelles was not assessed, the similarity in cytotoxicity suggests similar uptake of CTX between the two formulations.

To investigate the antitumor activity of MIF-sss-CTX, subcutaneous LLC tumor-bearing mice were treated with the same concentration of the Tween-80 based formulation or MIF-sss-CTX, through intraperitoneal injection. Compared with the control group, MIF-sss-CTX showed a similar therapeutic effect to the Tween-80 based formulation, both of

delayed tumor growth (**Figure 8A**) and prolonged overall survival time (**Figure 8B**), with no significant lasting toxicity effects based on reduced mouse body weight (**Figure 8C**).

Next, a refractory malignant pleural effusion (MPE) tumor model was used with LLC cells that were stably transfected with firefly luciferase. We performed bioluminescence imaging to detect the luciferase activity in LLC-Luc cells for the evaluation of pleural tumor burden. As shown in **Figure 8D**, both Tween-80 based formulation and MIF-sss-CTX treatments reduced pleural tumor burden. In addition, the total survival time of mice in the treatment group was prolonged (**Figure 8E**), with no obvious difference in body weight compared to PBS group (**Figure 8F**). Overall, MIF-sss-CTX and the Tween-80 based formulation provided similar anti-tumor responses. The impact of MIF itself on the tumor response was not assessed. Future studies that assess biodistribution and tumor drug levels would be useful to determine whether CTX accumulation is similar for these two formulations.

CONCLUSION

Elevated storage temperatures were used to optimize the type and amount of co-loader of stabilized, surfactant-stripped CTX micelles, resulting in a formulation stable in refrigerated aqueous storage for hundreds of days. MIF-sss-CTX exhibited reduced hemolysis and complement activation compared to a Tween-80 formulation, but had similar tubulin polymerization enhancement, pharmacokinetic profile, cytotoxicity and tumor inhibitory activity. Overall, these data confirm that stabilized, surfactant-stripped micelles are amenable for formulating CTX with long-term aqueous storage and anti-tumor activity.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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TABLES

Table 1 Composition of large-scale MIF-sss-CTX preparation.

Parameter	MIF-sss-CTX	Tween-80 CTX
Cabazitaxel (mg. mL^{-1})	3	40
Co-loader (mg. mL^{-1})	2.2 +/- 0.3	-
Surfactant (mg/mL)	9.7 +/- 0.8 (F127)	1090 (Tween-80)
Drug-to-surfactant ratio (mass)	1 : 3.2	1 : 27
Drug-to-surfactant ratio (molar)	4.7 : 1	0.06 : 1

Values show mean +/- std. dev. from 3 preparations

FIGURE LEGENDS

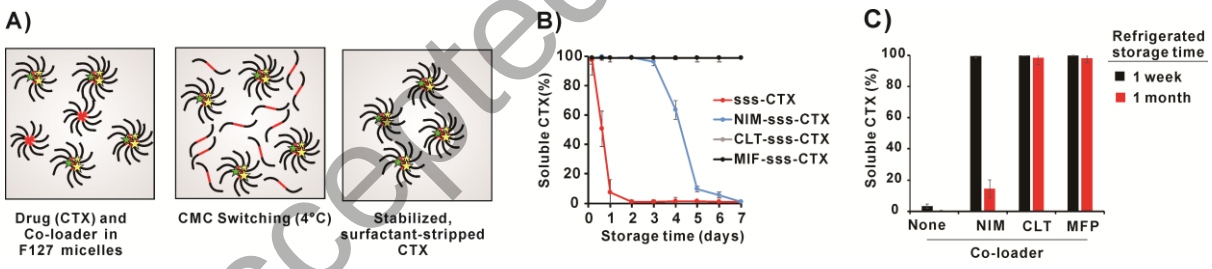

Fig. 1. Stability of stabilized, surfactant-stripped (sss) cabazitaxel (CTX) depends on the co-loader used. A) Schematic illustration of the sss-CTX preparation process by low-temperature surfactant stripping and use of a co-loader. **B)** Drug aggregation kinetics of sss-CTX micelles at room temperature, stabilized by mifepristone (MIF), clotrimazole (CLT), or nimodipine (NIM). **C)** Drug aggregation of sss-CTX micelles with various co-loaders stored at 4 °C. Mean +/- std. dev. for n=3 samples.

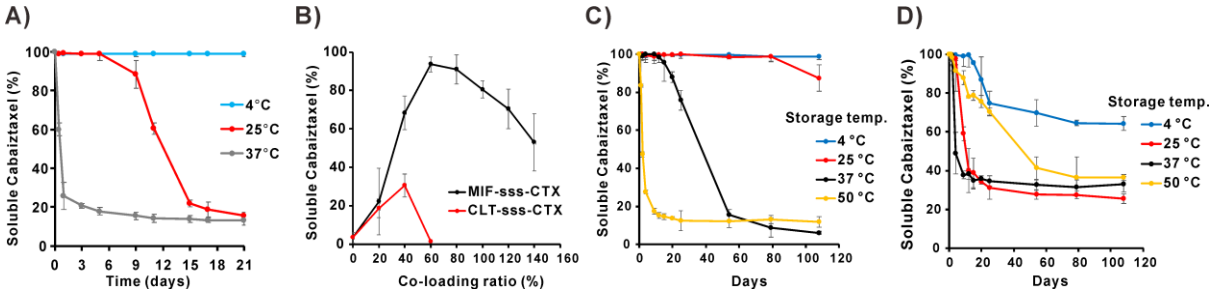

Fig. 2. Optimization of co-loader formulations in sss-CTX with storage at elevated temperatures. A) Illustration of aggregation kinetics of sss-CTX stabilized with ketoconazole (KTN), a previously identified inhibitor of sss-CTX aggregation with intermediate efficacy. KTN-sss-CTX was formed with 25 % mass ratio to CTX C. **B)** MIF or CLT were co-loaded into sss-CTX at the indicated ratios and micelles were stored at 37 °C for 15 days prior to assessing the amount of soluble drug. Storage stability of 5 mg/mL CTX under the indicated storage conditions in the form of MIF-sss-CTX (**C**), or a Tween-80 formulation (**D**). Values show mean +/- std. dev. for n=3 samples.

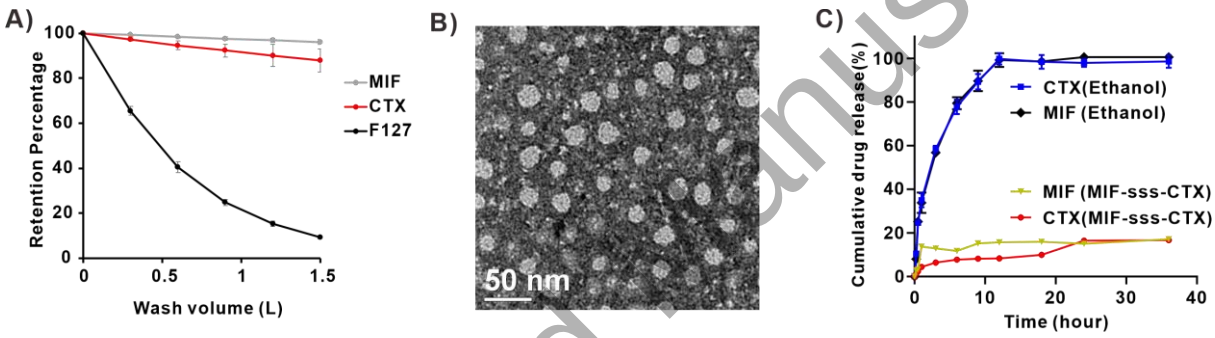

Fig. 3. A scalable formulation of MIF-sss-CTX A) Loss of each component of MIF-sss-CTX during large-scale tangential flow filtration washing at 4 °C. **B)** Negative-stained electron micrograph of MIF-sss-CTX micelles. **C)** In vitro drug release profiles of CTX formulations. MIF-sss-CTX and ethanol dissolved MIF or CTX were performed at 37 against 3% Tween 80 phosphate-buffered saline via dialysis membrane method. Mean +/- std. dev. for n=3 samples.

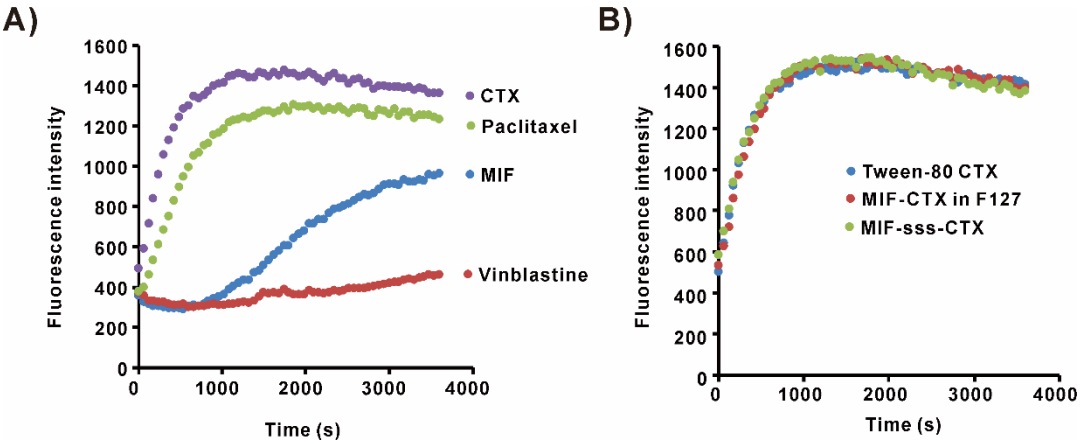

Fig. 4. Tubulin polymerization. Standard tubulin polymerization reaction rate was induced by various formulations at 10 μM drug concentration from a DMSO stock solution (**A)** or from indicated formulations (**B**).

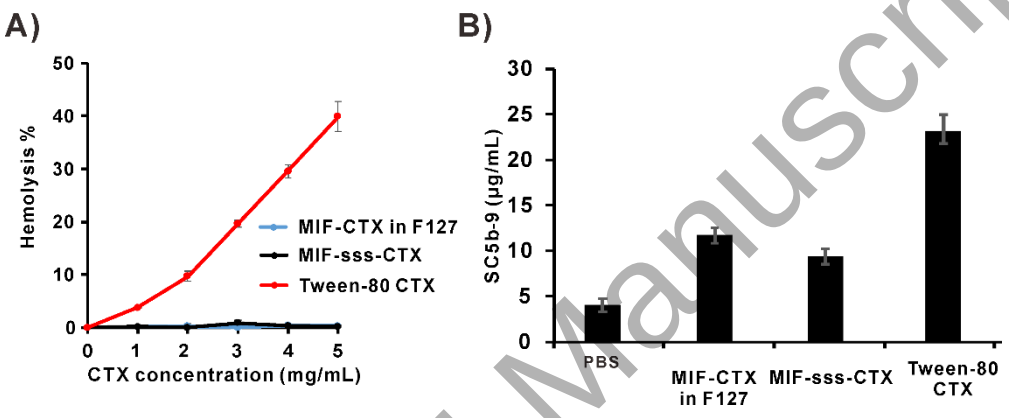

Fig. 5. Blood interaction with sss-CTX. A) Hemolysis activity following CTX incubation with human red blood cells in different CTX formulations. **B)** Complement activation assay with various CTX formulations at 1 mg/mL in fresh human plasma.

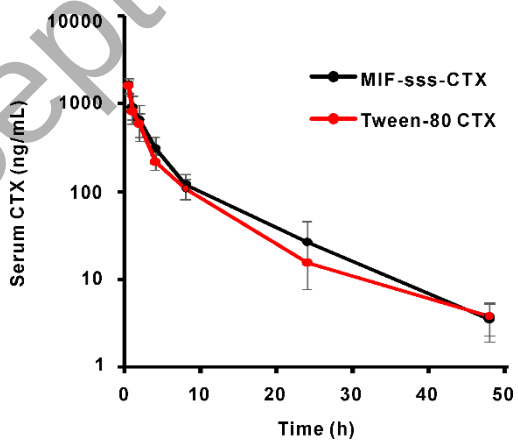

Fig. 6. Serum pharmacokinetic profile of CTX formulations. Serum pharmacokinetics of CTX in BALB/c mice after a single intravenous dose of 10 mg/kg (n=4 mice per group).

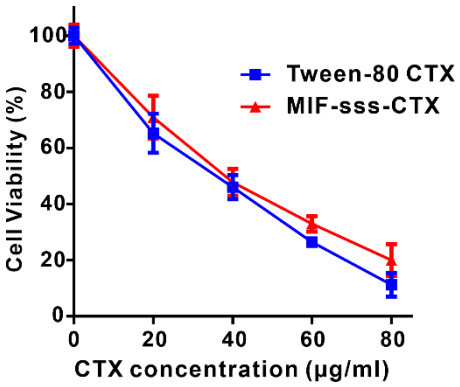

Fig. 7. In vitro cytotoxicity of cabazitaxel formulations in LLC cells. The antitumor efficacy against LLC cells was evaluated by a CCK-8 assay after a 24 hr incubation with CTX at indicated concentrations and formulations.

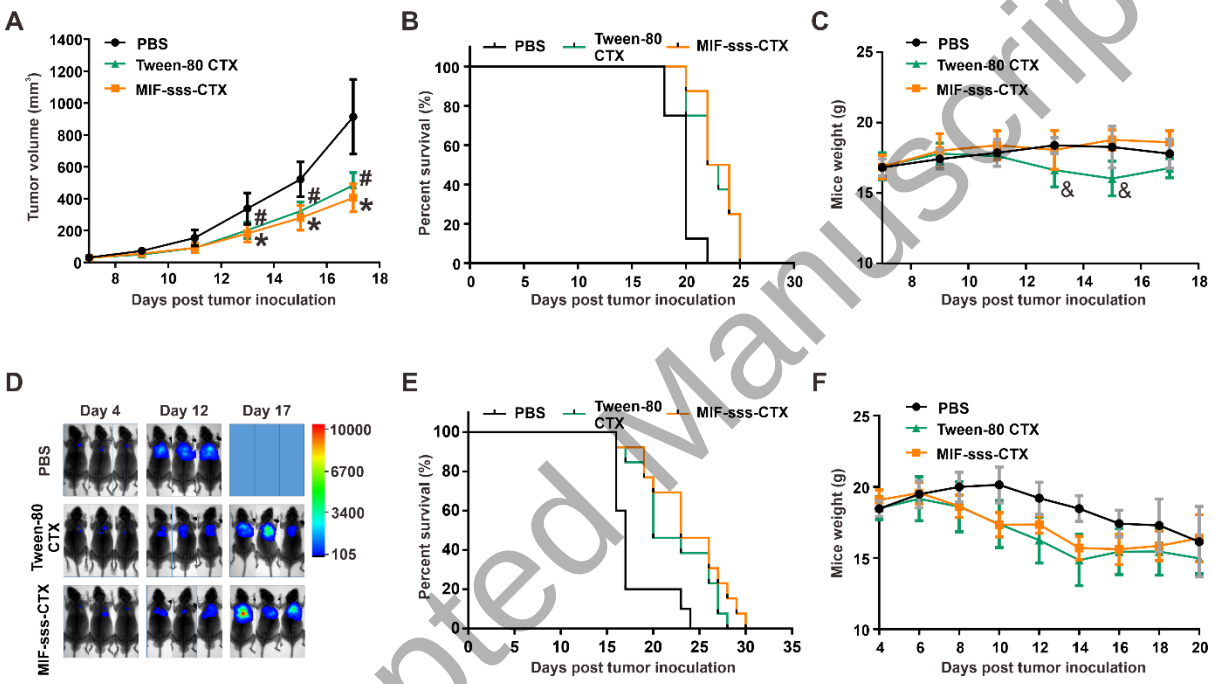

Fig. 8. Efficacy of CTX formulations in two murine Lewis lung carcinoma tumor models. Tumor volume, survival curve and mouse body weight in a subcutaneous LLC xenograft model (**A, B, C).** 3 injection of 6 mg kg^{-1} CTX on days 1, 3, 5 (n=8) were provided. For tumor growth curves, * represents MIF-sss-CTX group has significant difference and # represents Tween-80 based formulation group had a significant tumor volume difference, compared to the control group. & represents Tween-80 based formulation group has a significant weight difference with MIF-sss-CTX group ($p<0.05$, one-way ANOVA with Tukey's posthoc pairwise comparison). Bioluminescence imaging, survival curve and mouse bodyweight in a malignant pleural effusion LLC model (**D, E, F**). 3 injections of 8 mg kg^{-1} CTX were provided on days 1, 3, 5 (n=8).