International Journal of Nanomedicine

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ORIGINAL RESEARCH

Co-Delivery of Docetaxel and Curcumin Functionalized Mixed Micelles for the Treatment of Drug-Resistant Breast Cancer by Oral Administration

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Background: Chemotherapeutic drugs have some drawbacks in antineoplastic therapy, mainly containing seriously toxic side effects caused by injection and multi-drug resistance (MDR). Co-delivery with two or more drugs via nanomicelles is a promising strategy to solve these problems. Oral chemotherapy is increasingly preferred owing to its potential to enhance the life quality of patients.

Methods and Results: The study intended to develop mixed micelles using D-α-Tocopherol poly(ethylene glycol) 1000 succinate (TPGS) and soluplus for the co-encapsulation of docetaxel (DTX) and curcumin (CUR), marked as (DTX+CUR)-loaded mixed micelles, treating drug-resistant breast cancer by oral administration. The (DTX+CUR)-loaded mixed micelles had a uniform particle size (~64 nm), high drug loading and encapsulation efficiency, in vitro sustained-release properties and good pH-dependent stability. In vitro cell study, the (DTX+CUR)-loaded mixed micelles displayed the highest cellular uptake, cytotoxicity, cell apoptosis-inducing rates and cell ROS-inducing levels on MCF-7/Adr cells. Notably, in vivo pharmacokinetic studies, (DTX+CUR)-loaded mixed micelles enhanced markedly the oral absorption of DTX compared to pure DTX, with a relative oral bioavailability of 574%. The (DTX+CUR)-loaded mixed micelles by oral administration had the same anticancer efficacy as taxotere by injection in resistant breast cancer bearing mice.

Conclusion: (DTX+CUR)-loaded mixed micelles could provide a potential formulation for treating drug-resistant breast cancers by oral administration.

Keywords: docetaxel, mixed micelles, resistant breast cancer, oral delivery, bioavailability, mice

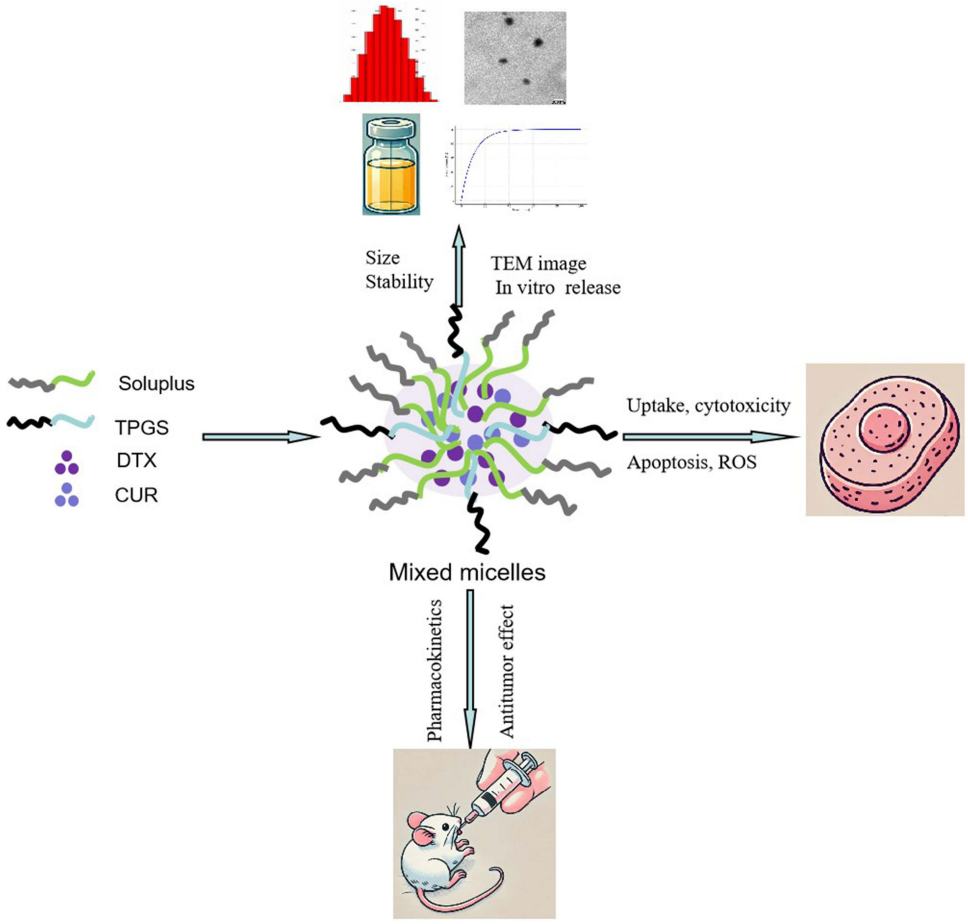
Introduction

Chemotherapy is pivotal in the clinical treatment of various tumors. Traditionally, many chemotherapeutic drugs are administered intravenously, leading to significant adverse reactions.[1] In contrast, oral chemotherapy offers numerous benefits, especially for chronic patients, including convenience, cost-effectiveness, pain alleviation, and improved quality of life.[2] It enables breast cancer patients to continue treatment at home after initial hospital care. However, the oral administration of most anticancer drugs is hindered by poor gastrointestinal absorption, largely due to the action of P-type glycoproteins (P-gp) in the intestine,[3,4] making the improvement of oral bioavailability a critical challenge for successful chemotherapy.

Docetaxel (DTX) is a widely used chemotherapeutic agent for treating various cancers.[5] Its clinical application is limited by poor water solubility and low oral bioavailability.[6] Administered intravenously with solvents like tween 80 and ethanol, DTX is associated with severe side effects such as neurotoxicity and neutropenia. The presence of P-gp efflux mechanisms in the gastrointestinal tract and metabolism by cytochrome P-4503A4 further restricts DTX's ability

Received: 6 April 2024
Accepted: 14 August 2024
Published: 22 August 2024

Graphical Abstract



to reach effective blood concentrations, highlighting the need for innovative approaches to develop oral formulations of DTX.[7–9] Moreover, DTX often faces challenges with multidrug resistance (MDR), primarily due to the overexpression of P-gp by the ABC transporter family.[10]

To counteract MDR, P-gp inhibitors such as verapamil and cyclosporine A have been explored, though their use is limited by potential immune system suppression.[11,12] Curcumin (CUR), a diphenol compound from *Curcuma longa*, has emerged as a promising P-gp inhibitor from traditional Chinese medicine, demonstrating safety up to doses of 12 g/day in humans and potential anti-MDR activity in various cancer cell lines.[13–16] However, its clinical application is limited by poor water solubility and low oral bioavailability.[17]

Polymeric micelles, capable of encapsulating hydrophobic drugs, offer a solution to improve solubility, prevent drug interaction with the intestinal membrane, enhance stability, and evade P-gp recognition.[18] These micelles, especially when designed as mixed micelles incorporating both TPGS and soluplus, could significantly enhance the oral bioavailability of hydrophobic drugs. TPGS, a water-soluble derivative of vitamin E, and soluplus, an amphiphilic copolymer, together facilitated drug permeability through cell membranes and inhibited P-gp, thereby enhancing oral drug absorption and mitigating multidrug resistance.[19,20]

The study introduced a mixed micelle formulation, (DTX+CUR)-loaded mixed micelles, for the co-delivery of DTX and CUR to address drug-resistant breast cancer through oral delivery. The formulation aimed to leverage the synergistic effects of DTX and CUR, supported by the physicochemical properties of TPGS and soluplus, to enhance cellular uptake,

cytotoxicity, apoptosis, and reactive oxygen species in MCF-7/Adr cells. Additionally, we explored the in vivo pharmacokinetics and anticancer efficacy of this nanomedicine approach, offering a potential breakthrough in treating drug-resistant breast cancers.

Materials and Method

Materials

Docetaxel (purity 99%, DTX) was sourced from Beijing Hvsf United Chemical Materials Co., Ltd. (China). Curcumin (purity 98%, CUR) and rhodamine 123 (Rh123) were obtained from Sigma-Aldrich (China). The amphiphilic polyvinyl caprolactam-polyvinyl acetate-polyethylene glycol graft copolymer (soluplus) was generously provided by BASF Auxiliary Chem. Co., Ltd. (Shanghai, China). D-α-Tocopherol poly(ethylene glycol) 1000 succinate (TPGS) was also purchased from Sigma-Aldrich (China). RPMI1640 medium was acquired from M&C Gene Technology (Beijing, China), and Fetal Bovine Serum was supplied by GIBCO, Invitrogen Corp. (USA). Chromatographic grade acetonitrile was from TJSHIELD Company (Tianjin, China), and all other reagents, of analytical grade, were used as received without further purification.

Preparation of Mixed Micelles

(DTX+CUR)-loaded mixed micelles were prepared by dissolving soluplus and TPGS at a 1/0.4 molar ratio, along with DTX and CUR at a 20:1 weight ratio of materials to drugs, in methanol. The methanol was then evaporated at 37°C using a vacuum rotary evaporator to form a thin film. This film was hydrated with deionized water and stirred magnetically for 2 hours at room temperature to obtain a micellar solution. The (DTX+CUR)-loaded mixed micelles were achieved by filtrating through a 0.22 μm polycarbonate membrane to remove the unencapsulated drugs.[21] Other micelles were prepared using the same procedures as described above. Similarly, Rh123 mixed micelles were prepared for use as fluorescent probes.

Measurements of Particle Size and Zeta Potential

The particle size, zeta potential, and polydispersity index (PDI) of the micelles were determined by a Zetasizer Nano ZS-90 (Malvern, UK). The morphology was examined by transmission electron microscopy (TEM, JEOL, JEM-200 CX, Japan).

Measurements of Encapsulation Efficiency and Drug Loading

Encapsulation efficiency (EE) and drug loading (DL) were measured by high-performance liquid chromatography (HPLC, Agilent Technologies Inc, USA) with a reverse-phase C-18 column at 230 nm. The mobile phase consisted of acetonitrile and water (47/53, V/V) at a flow rate of 1 mL/min. Then CUR content was also analyzed by HPLC with a mobile phase made up of methanol, water, and acetic acid (76:23:1, v/v/v) at the wavelength of 421 nm. A flow rate was set at 1.0 mL/min. The encapsulation efficiency (EE) and drug loading (DL) were calculated using the formula: EE (%) = $(W_1/W_2) \times 100\%$ and DL (%) = $(W_1/W_3) \times 100\%$, where W_1 is the encapsulated drug amount, W_2 is the total drug amount, and W_3 is the total weight of drug and carrier.[22]

In vitro Characterization

The in vitro release profile of the drug from the micelles was evaluated using a dialysis method.[23] A volume of 1mL micelles containing 100 μg of drug was placed into a dialysis bag (MWCO 12–14 KDa, Greenbird Inc., Shanghai) and subjected to dialysis against 100 mL of simulated gastric fluid (SGF, pH 1.2) for 2 hours and then against simulated intestinal fluid (SIF, PBS pH 7.4) for up to 48 hours, both at 37 ± 0.5°C with 100 revolutions per minute. At specific time intervals, a volume of 1 mL release medium was replaced with isothermal fresh medium. A suspension of DTX or CUR in water served as the control. Drug content was analyzed by HPLC. Each experiment was repeated three times for consistency.

PH-Dependent Stability

The pH-dependent stability of (DTX+CUR)-loaded mixed micelles was assessed in simulated gastrointestinal fluids at varying pH levels (1.2, 3.5, and 7.4). Briefly, 2 mL of the micelle dispersion was incubated with 10 mL of each simulated fluid. The incubation times were set at 2 hours for pH 1.2 and 3.5 and extended to 6 hours for pH 7.4. After incubation, particle size and polydispersity index (PDI) were measured, providing insights into the stability of the micelles under different gastrointestinal conditions.[24]

Cell Culture

MCF-7 and MCF-7/Adr cells were obtained from Guangdong Bozhihuihai Biotechnology Co., LTD (Dongguan, China) and cultured in RPMI 1640 medium including 10% fetal bovine serum under an atmosphere of 5% CO_2 at 37°C. To ensure the maintenance of drug resistance, MCF-7/Adr cells were cultured in a low concentration of DTX (1 μM) and passaged in a drug-free medium for one week before experiments. All cell lines used in this study were approved by the Ethics Committee of Guangdong Medical University.

Cytotoxicity Assessment

The molar ratio of the two drugs in the co-delivery system significantly impacted the therapeutic outcome. To determine the optimal DTX/CUR molar ratio, MCF-7/Adr cells were utilized. These cells were plated at a density of 5×10^3 cells per well in 96-well plates and incubated for 24 hours. Following the initial incubation, the culture medium was replaced with (DTX+CUR)-loaded mixed micelles at various molar ratios (DTX/CUR in mixed micelles = 1.0/0, 0/1.0, 0.8/0.2, 0.5/0.5, 0.2/0.8). The total concentration of drug in these formulations was maintained at 5 μM. After further 48-hour incubation, cell viability was assessed using sulforhodamine B (SRB) assays to estimate the optimal DTX/CUR molar ratio.[25]

Cytotoxicity was evaluated on MCF-7 and MCF-7/Adr cells seeded at 5×10^3 cells per well in 96-well plates and incubated for 24 hours. The cells were treated with various formulations, including pure CUR, pure DTX, pure (DTX+CUR), DTX-loaded-micelles, DTX-loaded mixed micelles, and (DTX+CUR)-loaded mixed micelles, for 48 hours. The final concentration of DTX in dual-drug formulation was half that of single-drug formulation. The drug-free medium was utilized as control. The cells were determined using sulforhodamine B (SRB) assays after incubation for 48 h. The absorbance was measured at 540 nm using Cytation5 cell imaging multifunctional detector. Cell viability was calculated based on the optical density (OD) values using the following formulation: Survival rate % = ($OD_{540\ nm}$ for the treated cells/$OD_{540\ nm}$ for the control cells) × 100%, where $OD_{540\ nm}$ is the optical density value. Dose-effect curves were generated.

Cellular Uptake

The uptake of formulations by MCF-7/Adr cells was qualitatively observed using a laser scanning confocal microscope (Leica SP2, Heidelberg, Germany) and quantitatively analyzed by flow cytometry. Rhodamine 123 (Rh123), serving as a fluorescent surrogate for DTX due to its P-gp substrate property, was utilized in the study. MCF-7/Adr cells were seeded into the polystyrene cell culture dishes at a density of 3×10^5 cells per dish under 5% CO_2 at 37°C for 24 h. Then, cells were incubated with pure Rh123, pure (Rh123+CUR), Rh123-loaded micelles, Rh123-loaded mixed micelles, and (DTX+CUR)-loaded mixed micelles for 4 hours. The concentration of Rh123 in the single-drug formulation was maintained at 10 μM, while in the dual-drug formulations, both Rh123 and CUR were used at 5 μM each. The drug-free medium was utilized as control. For qualitative study, after incubation for 4 hours, cell culture medium was discarded, and cells were washed three times with cold PBS. Cells were stained with Hoechst 33342 (10 μg/mL) for 20 minutes to visualize nuclei and analyzed for Rh123 uptake, and then washed three times again with cold PBS. Finally, MCF-7/Adr cells were observed using the laser scanning confocal microscope (excitation wavelength = 488 nm, emission wavelength = 560 nm). For quantitative study, after incubation for 4 hours, cells were washed three times with cold PBS, and then trypsinized with 0.25% trypsinase and collected. The obtained cells were analyzed using flow cytometry to determine the fluorescence intensity of Rh123 for estimating the amount of cellular uptake (excitation wavelength = 488 nm, emission

wavelength = 560 nm). The two approaches facilitated a comprehensive understanding of the cellular internalization of the micelles.

Apoptosis Induction

The induction of apoptosis in MCF-7/Adr cells by different formulations was quantitatively estimated by flow cytometry. MCF-7/Adr cells were seeded into 6-well plates at a density of 3×10^5 cells per well under 5% CO_2 at 37°C for 24 h. Then, cells were treated for 16 h with pure DTX, pure (DTX+CUR), DTX-loaded micelles, DTX-loaded mixed micelles, (DTX+CUR)-loaded mixed micelles, respectively. The concentration of DTX in the single-drug formulation was maintained at 10 μM, while in the dual-drug formulations, both DTX and CUR were used at 5 μM each. The drug-free medium was utilized as control. After incubation for 16 hours, the cells were washed twice with PBS, trypsinized, collected, and resuspended in 300 μL of binding buffer supplied in the test kit. Then, cells were stained with 5 μL AnnexinV-KFluor647 and 5 μL 7-AAD in the dark at room temperature for 10 minutes. Finally, cells were analyzed to determine the extent of apoptosis.

Intracellular ROS Detection

The production of intracellular reactive oxygen species (ROS) was quantitatively detected by flow cytometry. MCF-7/Adr cells were seeded into 6-well plates at a density of 3×10^5 cells per well under 5% CO_2 at 37°C for 24 hours. Then cells were treated with pure DTX, pure (DTX + CUR), DTX-loaded micelles, DTX-loaded mixed micelles, (DTX +CUR)-loaded mixed micelles, respectively. The concentration of DTX in the single-drug formulation was maintained at 10 μM, while in the dual-drug formulations, both DTX and CUR were used at 5 μM each. The drug-free medium was utilized as control. After incubation for 18 hours, mediums were discarded. Then, Cells were incubated with DCFH-DA (10 μM) for 20 minutes in the dark, trypsinized and collected. Finally, cells were analyzed to evaluate ROS levels by flow cytometry (excitation wavelength = 488 nm, emission wavelength = 525 nm).

Pharmacokinetic Study

The experiment aimed to compare the in vivo absorption of (DTX+CUR)-loaded mixed micelles with pure DTX by oral administration. Female Sprague-Dawley rats, weighing approximately 200 ± 20 g, were obtained from the Guangdong Medical Laboratory Animal Center in China. All experimental procedures complied with the guidelines for the care and use of laboratory animals and were approved by the Administrative Committee on Animal Research at Guangdong Medical University (approval No. 2023–0099). Before the study, rats were acclimated to a controlled environment with standard feed and water for one week and were fasted overnight. They were then divided into two groups, each consisting of six rats: Group I was administered a 20 mg/kg dose of pure DTX in distilled water with 0.3% CMC-Na orally;[26] Group II was given an oral (DTX+CUR)-loaded mixed micelles solution at an equivalent dose of 20 mg/kg, consisting of 13.74 mg/kg for DTX and 6.26 mg/kg for CUR. Blood samples were subsequently collected from the eye vein plexus into heparinized tubes at specified intervals: 0.0, 0.5, 1.0, 2.0, 3.0, 4.0, 8.0, 12.0, 24.0, and 36.0 hours post-administration. Plasma was separated by centrifugation at 5000 rpm for 10 minutes and stored at −20°C until analyzed.

To analyze the plasma DTX concentration, a 100 μL of the plasma sample was mixed with 1 mL of t-butyl methyl ether. This mixture was then vortexed for 3 minutes and centrifuged at 12,000 rpm for 15 minutes. The supernatant was transferred to a new tube and evaporated under vacuum at room temperature overnight. The residue was reconstituted in 100 μL of methanol and vortexed for 5 minutes, followed by centrifugation at 12,000 rpm for 15 minutes at room temperature. An 80 μL aliquot of the supernatant was then subjected to analysis using an HPLC system. The system was equipped with an Odyssil C_{18} column (4.6 × 250 mm, 5 μm) and a guard column (4.6 × 12.5 mm, 5 μm), operating at a detection wavelength of 230 nm. The mobile phase consisted of a 47/53 mixture of acetonitrile and water, with a flow rate set at 1.0 mL/min.

Pharmacokinetic analysis for each formulation was performed using DAS software (version 2.0). The maximum plasma concentration of docetaxel (C_{max}) and the time to reach this concentration (T_{max}) were obtained directly from the experimental data. The half-life ($t_{1/2}$) was calculated using the formula 0.693/Ke. The mean residence time (MRT) and the area under the curve from time zero to infinite ($AUC_{0-\infty}$) were determined using the linear trapezoidal rule.

Anticancer Efficacy

Female nude mice (BALB/c, weighing 18–20 g) were acquired from the Guangdong Medical Laboratory Animal Center, China. This study adhered to the established guidelines and received approval from the Administrative Committee on Animal Research at Guangdong Medical University (approval No. 2023-0099). The mice were injected subcutaneously with MCF-7/Adr cells, and their growth was monitored until the tumor volumes reached approximately 150 mm^3. Subsequently, the mice were divided into five groups (each containing 6 mice): the control group received saline orally (p.o)., the pure DTX group received 10 mg/Kg (p.o)., the taxotere group received 10 mg/Kg intravenously (i.v)., the DTX-loaded micelles group received 10 mg/Kg (p.o)., and the (DTX+CUR)-loaded mixed micelles group received 6.87 mg/Kg for DTX and 3.13 mg/Kg for CUR (p.o). Treatments were conducted every two days for a total of 6 doses. The assessment of anticancer effects was based on the recording of tumor volume and body weight.

Statistical Analysis

Data analysis utilized SPSS 22.0 software. A one-way ANOVA is tested for significance ($p < 0.05$), with results presented as mean ± standard deviation (SD).

Results

Characterization of Micelles

A schematic diagram of mixed micelles formation is shown in Figure 1A. The characterization of the nanomicelles was detailed by measuring average particle sizes, PDI, zeta potential, drug loading, and encapsulation efficiency, as shown in

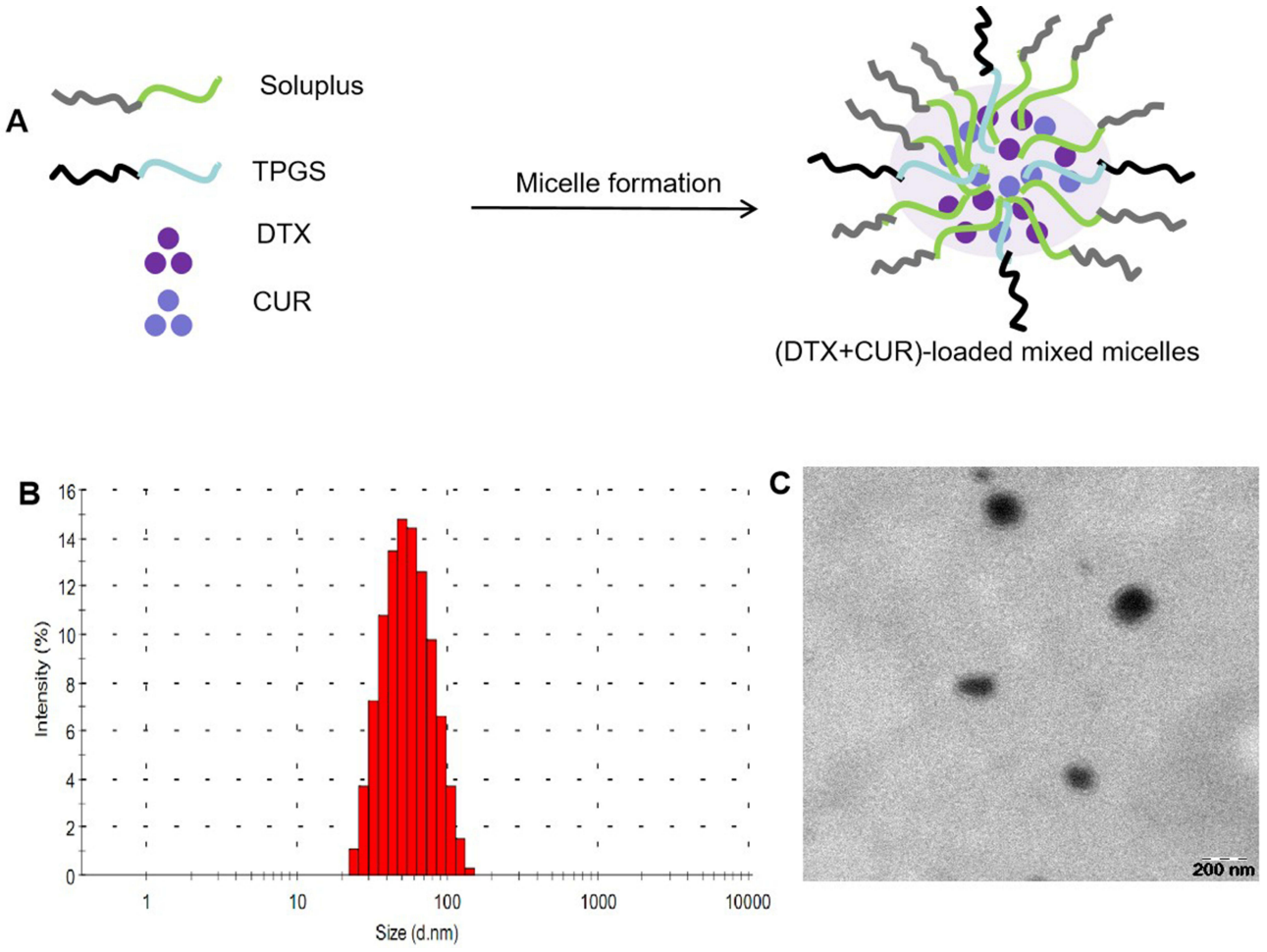


Figure 1 Characterization of (DTX+CUR)-loaded mixed micelles. (**A**) Schematic representations of the formulation. (**B**) Size distribution. (**C**) TEM image.

Table 1 Characterization of Mixed Micelles (Mean ± SD, n = 3)

Sample		Blank Mixed Micelles	DTX-Loaded-Micelles	DTX-Loaded Mixed Micelles	(DTX+CUR)-Loaded Mixed Micelles
Size (nm)		57.15 ± 0.13	58.57 ± 0.16	61.64 ± 0.14	63.80 ± 0.15
PDI		0.10±0.01	0.12±0.01	0.11±0.02	0.10±0.02
Zeta (mV)		−16.41 ± 1.38	−17.20 ± 1.53	−20.38 ± 1.91	−24.10 ± 1.80
DTX	EE (%)	-	97.68 ± 2.72	95.07 ± 3.61	92.08 ± 2.14
	DL (%)	-	5.18 ± 0.14	5.67 ± 0.21	3.54 ± 0.12
CUR	EE (%)	-	-	-	94.73 ± 3.47
	DL (%)	-	-	-	1.59 ± 0.13

Abbreviations: DTX, docetaxel; CUR, curcumin; PDI, polydispersity index; DL, drug loading; EE, encapsulation efficiency. Micelles were formed using soluplus and mixed micelles were formed using soluplus and TPGS, TPGS, tocopherol acid polyethylene glycol succinate;

Table 1. The results revealed that the average particle size of the (DTX+CUR)-loaded mixed micelles was approximately ~64 nm (Figure 1B), with a narrow size distribution indicated by a PDI of less than 0.20. Transmission electron microscopy (TEM) images further confirmed that the (DTX+CUR)-loaded mixed micelles were uniformly spherical (Figure 1C). The zeta potential measurements for the (DTX+CUR)-loaded mixed micelles were −24.10 ± 1.80 mV. Additionally, the drug loading efficiency for all micelle formulations exceeded 5%, and the encapsulation efficiencies were greater than 90%.

The release rates of drugs from various formulations were examined in simulated gastric fluid (SGF) over 2 hours and in simulated intestinal fluid (SIF) for up to 48 hours, as illustrated in Figure 2. The cumulative release of DTX from pure DTX, DTX-loaded micelles, DTX-loaded mixed micelles, and (DTX+CUR)-loaded mixed micelles was found to be roughly 14.13 ± 1.83%, 5.03 ± 2.67%, 6.04 ± 2.74%, and 4.32 ± 2.08% in SGF for 2 hours, respectively, and 22.59 ±

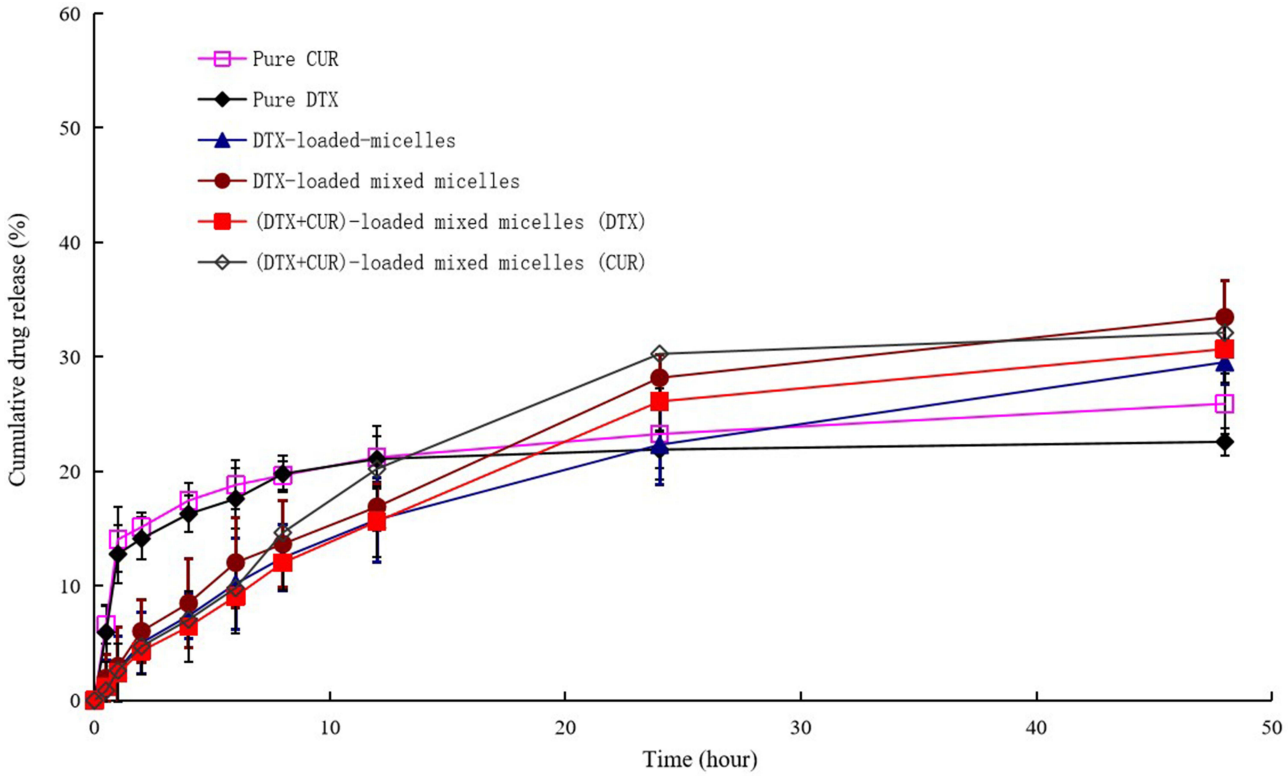


Figure 2 The in vitro drug release behavior of different formulations in simulated gastric fluid (pH 1.2 for 2 hours) and simulated intestinal fluid (pH 7.4 up to 48 hours). Data are presented as mean ± SD (n=3).

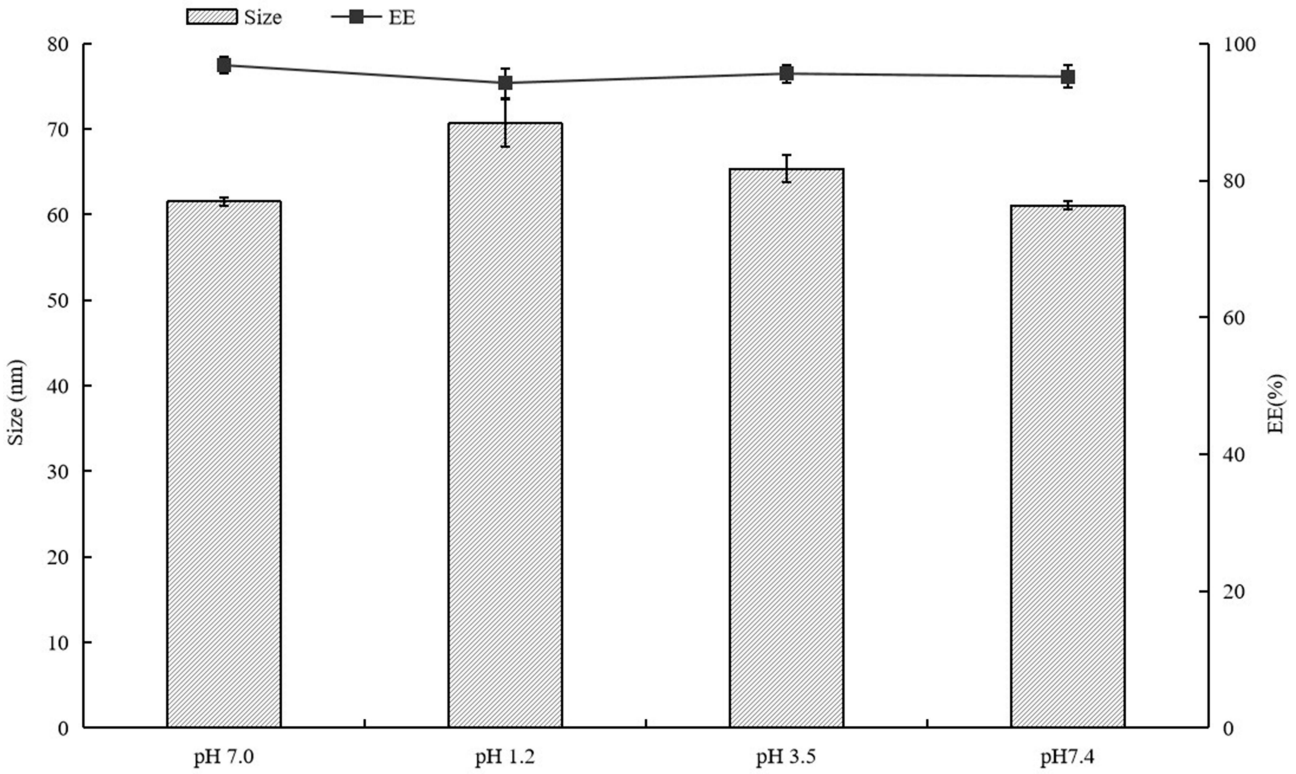


Figure 3 The stability of (DTX+CUR)-loaded mixed micelles in different simulated GIT fluids (pH 1.2 and pH 3.5 for 2 hours, pH 7.4 for 2 hours). PH7.0 was utilized as the control. Data are presented as mean ± SD (n=3).

1.17%, 29.54 ± 1.95%, 33.47 ± 3.14%, and 30.69 ± 2.90% in SIF for over 48 hours, respectively. Similarly, the cumulative release of CUR from pure CUR and (DTX+CUR)-loaded mixed micelles was about 15.34 ± 2.75%, 6.42 ± 2.81% in SGF for 2 hours, and 25.91 ± 2.62%, 32.11 ± 2.23% in SIF for over 48 hours, respectively. These findings highlighted a sustained release pattern of DTX and CUR from the (DTX+CUR)-loaded mixed micelles, which released less than 35% over 48 hours. Remarkably, the release rates of DTX and CUR from the (DTX+CUR)-loaded mixed micelles were very similar, underscoring their potential for sustained drug delivery.

The stability of (DTX+CUR)-loaded mixed micelles was assessed in artificial gastrointestinal fluids at various pH levels (1.2, 3.5, and 7.4), with results shown in Figure 3. The particle size of (DTX+CUR)-loaded mixed micelles varied minimally, from 61.54 ± 0.49 nm to 70.72 ± 2.77 nm at pH 1.2 for 2 hours, 65.38 ± 1.64 nm at pH 3.5 for 2 hours, and 61.07 ± 0.47 nm at pH 7.4 for 6 hours, while maintaining a PDI below 0.2. These findings demonstrated the formulations' stable nature in simulated gastrointestinal conditions, facilitating drug absorption.

Cytotoxicity

Figure 4 presents the cell viability rate of MCF-7/Adr cells after 48 hours of treatment with samples of different DTX/CUR molar ratios in mixed micelles (1.0/0, 0/1.0, 0.2/0.8, 0.5/0.5, 0.8/0.2) at various concentrations. Clearly, the sample with a DTX/CUR ratio of 0.5/0.5 in mixed micelles showed significantly higher cytotoxicity compared to the other samples.

The cytotoxic effects of different DTX formulations on MCF-7 and MCF-7/Adr cells were evaluated using the Sulforhodamine B (SRB) assay, as illustrated in Figure 5. Blank mixed micelles made from TPGS and soluplus showed lower cytotoxicity to both MCF-7 and MCF-7/Adr cells. The cytotoxic impact of all formulations on MCF-7 and MCF-7/Adr cells was found to be dependent on the concentration of DTX. The cytotoxicity ranking was as follows: (DTX+CUR)-loaded mixed micelles > DTX-loaded mixed micelles > DTX-loaded micelles > pure DTX > pure (DTX+CUR) > Blank mixed micelles. Pure DTX showed significant cytotoxicity towards MCF-7 cells but was minimally effective

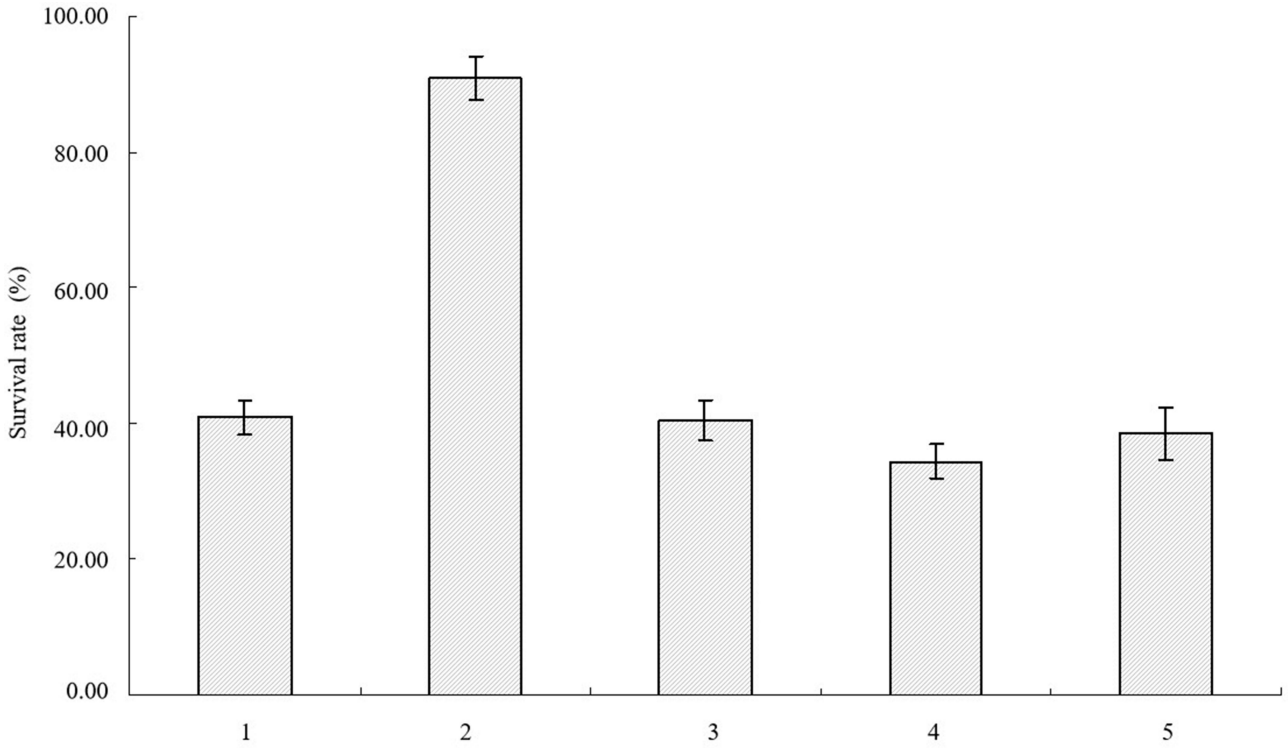


Figure 4 Survival rates of MCF-7/Adr cells treated with (DTX+CUR)-loaded mixed micelles at various molar ratios of DTX/CUR respectively for 48 h.
Notes: 1, 1:0; 2, 0:1; 3, 0.8:0.2; 4, 0.5:0.5; 5, 0.2:0.8. Data are presented as mean ± SD (n = 3).

against MCF-7/Adr cells. However, formulations containing DTX micelles significantly enhanced DTX's cytotoxicity against MCF-7/Adr cells. Notably, even with only half the DTX concentration compared to the single-drug formulations, the (DTX+CUR)-loaded mixed micelles displayed the highest cytotoxicity towards MCF-7/Adr cells. The indicated that curcumin (CUR) significantly boosted the effectiveness of DTX against drug-resistant cancer cells.

Cellular Uptake

The cellular uptake of different formulations by MCF-7/Adr cells was qualitatively evaluated using a laser scanning confocal microscope, with Rh123 serving as a fluorescent marker for DTX. In this setup, green fluorescence represented the internalization of Rh123 by the cells, and blue fluorescence identified the cell nuclei with Hoechst 33342. Figure 6A showed that the pure Rh123 exhibited the lowest fluorescence intensity within MCF-7/Adr cells. In contrast, the (DTX+CUR)-loaded mixed micelles formulation, even with Rh123 concentration at only half of that in the formulations containing solely Rh123, showed the highest intensity. This indicated that the formulation significantly enhanced cellular uptake.

Quantitative analysis of cellular uptake, depicted in Figure 6B and C and validated by flow cytometry, revealed the levels of Rh123 uptake through mean fluorescence intensity. These intensities were recorded as 561.93 ± 8.68 for the control, 9787.43 ± 184.35 for pure Rh123, 5613.16 ± 185.79 for pure (Rh123 + CUR), 19,441.83±1058.27 for Rh123-loaded micelles, 540931.22±18,505.96 for Rh123-loaded mixed micelles, and 542056.58 ± 9006.73 for (Rh123 + CUR)-Loaded mixed micelles, respectively. These findings aligned with the qualitative cellular uptake observations made using the confocal laser scanning microscope.

Cell Apoptosis

Figure 7 displays the effect of inducing apoptosis after treating MCF-7/Adr cells with various formulations. Apoptosis rates in MCF-7/Adr cells after treating with blank culture medium, pure DTX, pure (DTX+CUR), DTX-loaded micelles, DTX-loaded mixed micelles and (DTX+CUR)-loaded mixed micelles were 2.19 ± 0.42%, 4.30 ± 0.94%, 3.93 ± 1.01%, 5.28 ± 1.25%, 59.75 ± 3.85% and 60.97 ± 3.14%, respectively. Notably, the (DTX+CUR)-loaded mixed micelles

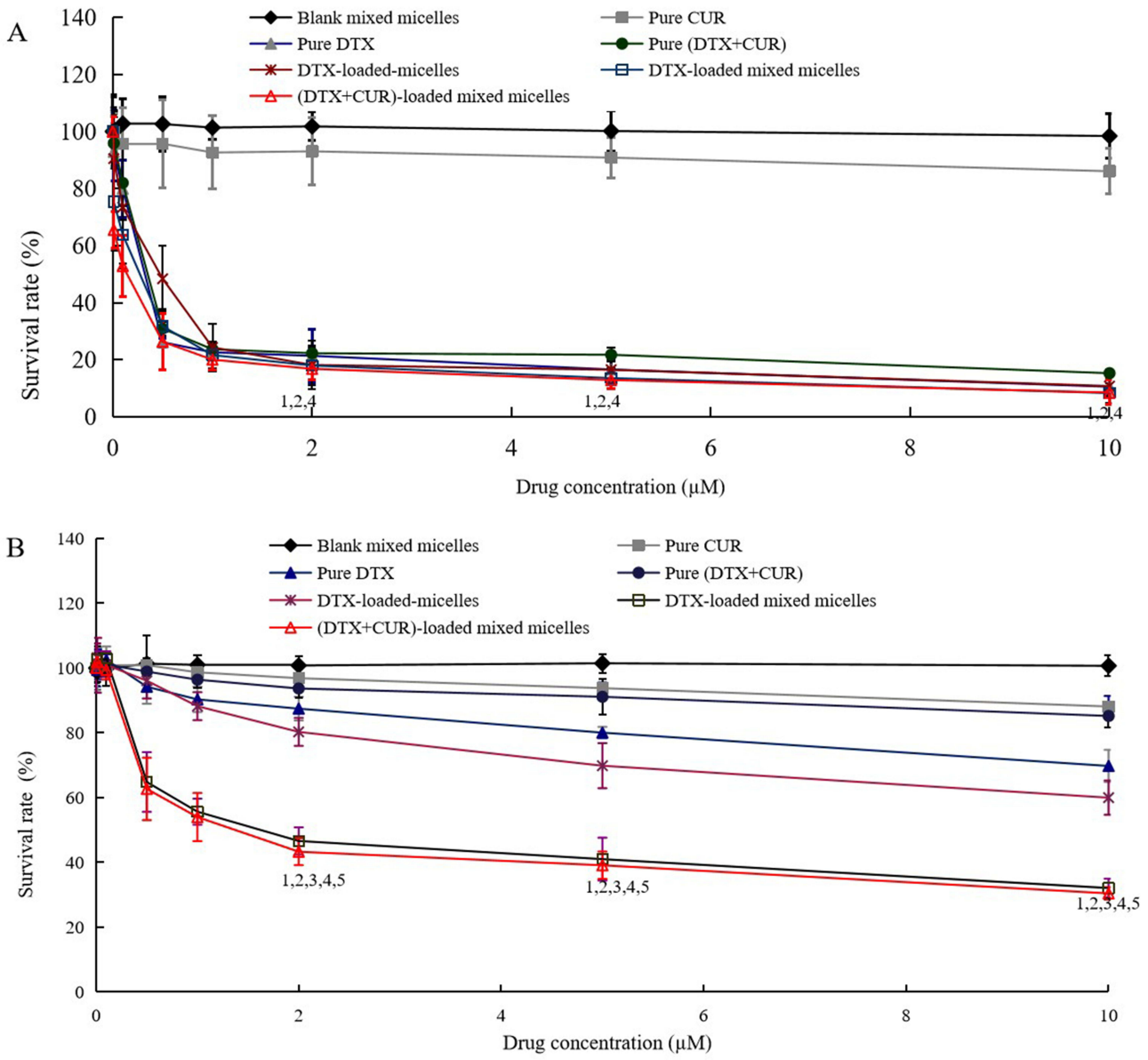


Figure 5 Survival rates of MCF-7 (**A**) or MCF-7/Adr (**B**) cells treated with various formulations respectively for 48 h.
Notes: 1, blank mixed micelles; 2, pure CUR; 3, pure DTX; 4, pure (DTX+CUR); 5, DTX-loaded-micelles; 6, DTX-loaded mixed micelles; 7, (DTX+CUR)-loaded mixed micelles. P, 0.05. Data are presented as mean ± SD (n = 3).

formulation, despite having only half the DTX concentration compared to the formulations with DTX alone, showed greater lethality than other treatments to MCF-7/Adr cells. The results indicated that (DTX+CUR)-loaded mixed micelles enhanced significantly the apoptosis-inducing effects on MCF-7/Adr cells, indicating increased therapeutic efficacy.

Detection of Intracellular Reactive Oxygen Species (ROS)

Figure 8 demonstrates the induction of reactive oxygen species (ROS) in MCF-7/Adr cells by various formulations. The data showed that the formulation of (DTX+CUR)-loaded mixed micelles exhibited the highest ROS levels, followed by DTX-loaded mixed micelles, DTX-loaded micelles, pure DTX, pure (DTX+CUR), and finally, the blank control. Notably, the (DTX+CUR)-loaded mixed micelles formulation, despite having only half the DTX concentration compared to the formulations with DTX alone, was the most effective at inducing ROS production in MCF-7/Adr cells. The activation of the ROS pathway could potentially lead to the apoptosis of MCF-7/Adr cells.

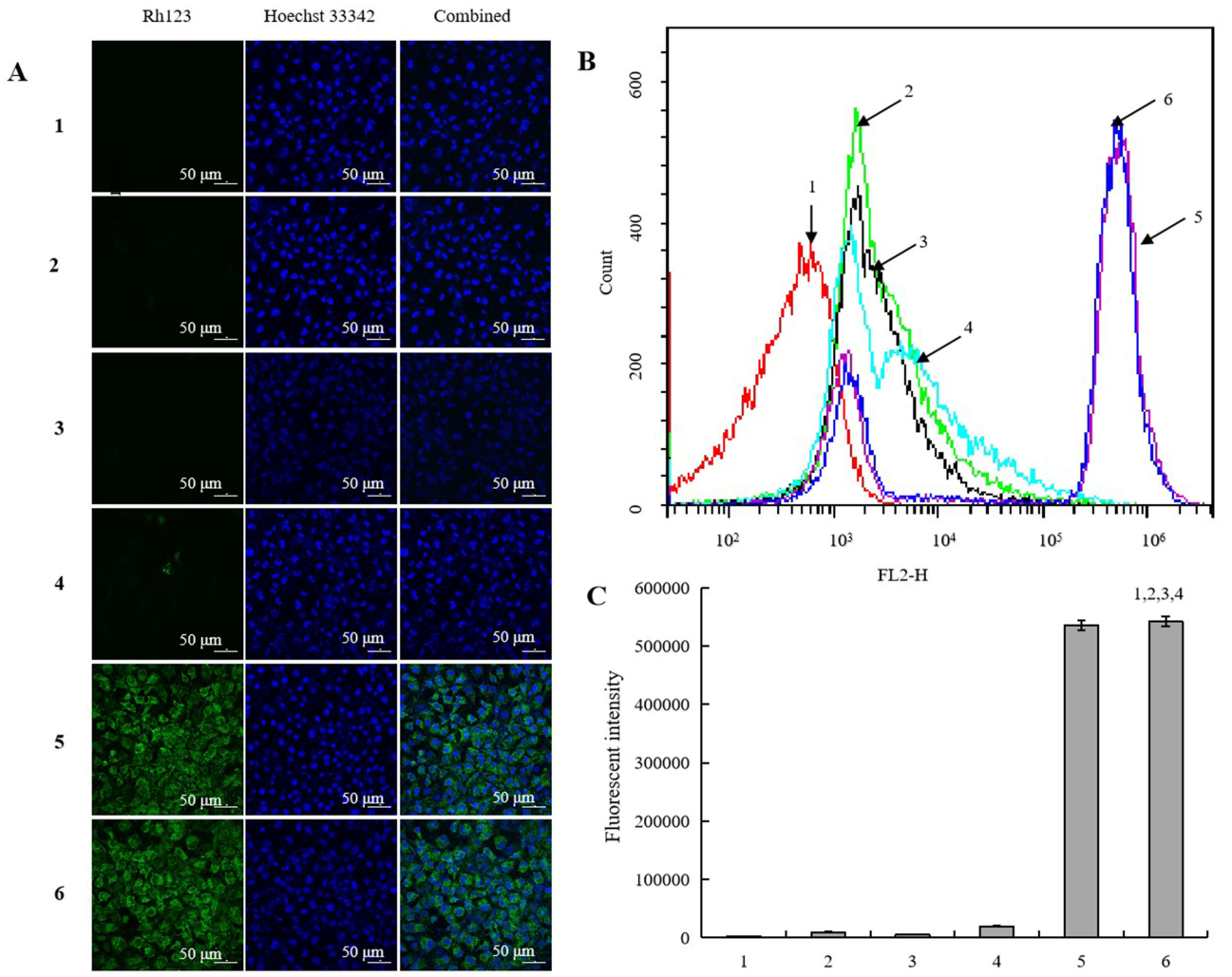


Figure 6 Intracellular uptake by MCF-7/Adr cells after treatment with various formulations. (**A**) Laser scanning confocal fluorescence images of MCF-7/Adr cells. Rh123 was used as a fluorescent indicator, while the nucleus was stained with Hoechst 333342; (**B** and **C**) Flow cytometry analysis of intracellular uptake by MCF-7/Adr cells: 1, control; 2, pure Rh123 (10 μM); 3, pure (Rh123+CUR) (5 μM for Rh123; 5 μM for CUR); 4, Rh123-loaded-micelles (10 μM); 5, Rh123-loaded mixed micelles (10 μM); 6, (Rh123+CUR)-loaded mixed micelles (5 μM for Rh123; 5 μM for CUR). $P < 0.05$. Data are presented as mean ± SD (n=3).

Pharmacokinetic Study

The oral bioavailability of (DTX+CUR)-loaded mixed micelles was estimated in Sprague-Dawley rats in comparison with that of pure DTX. The pharmacokinetic parameters were summarized in Table 2 and the mean plasma concentration-time curves of DTX after treatment with different formulations were drawn in Figure 9. The formulation of (DTX +CUR)-loaded mixed micelles could markedly increase the plasma half-time ($T_{1/2}$), peak concentration (C_{max}), area under the curve ($AUC_{0-\infty}$) and mean retention time (MRT) values in comparison with that of pure DTX. What's more, the plasma concentration of DTX for (DTX+CUR)-loaded mixed micelles continued for over 36 hours, while that of DTX in pure DTX reduced below the detect ability for 8 hours. Moreover, the $T_{1/2}$ of (DTX+CUR)-loaded mixed micelles was enhanced by 5.95 compared to that of pure DTX. The MRT of (DTX+CUR)-loaded mixed micelles was prolonged by 5.29 times compared to that of pure DTX. The result showed mixed micelles could prolong the retention time of drugs in the blood circulation system. According to the $AUC_{0-\infty}$values, the relative oral bioavailability of (DTX +CUR)-loaded mixed micelles was approximately 574% compared to that of pure DTX. In short, the (DTX+CUR)-loaded mixed micelles formulation possessed higher relative oral bioavailability, suggesting that it significantly promoted the oral absorption of drugs.

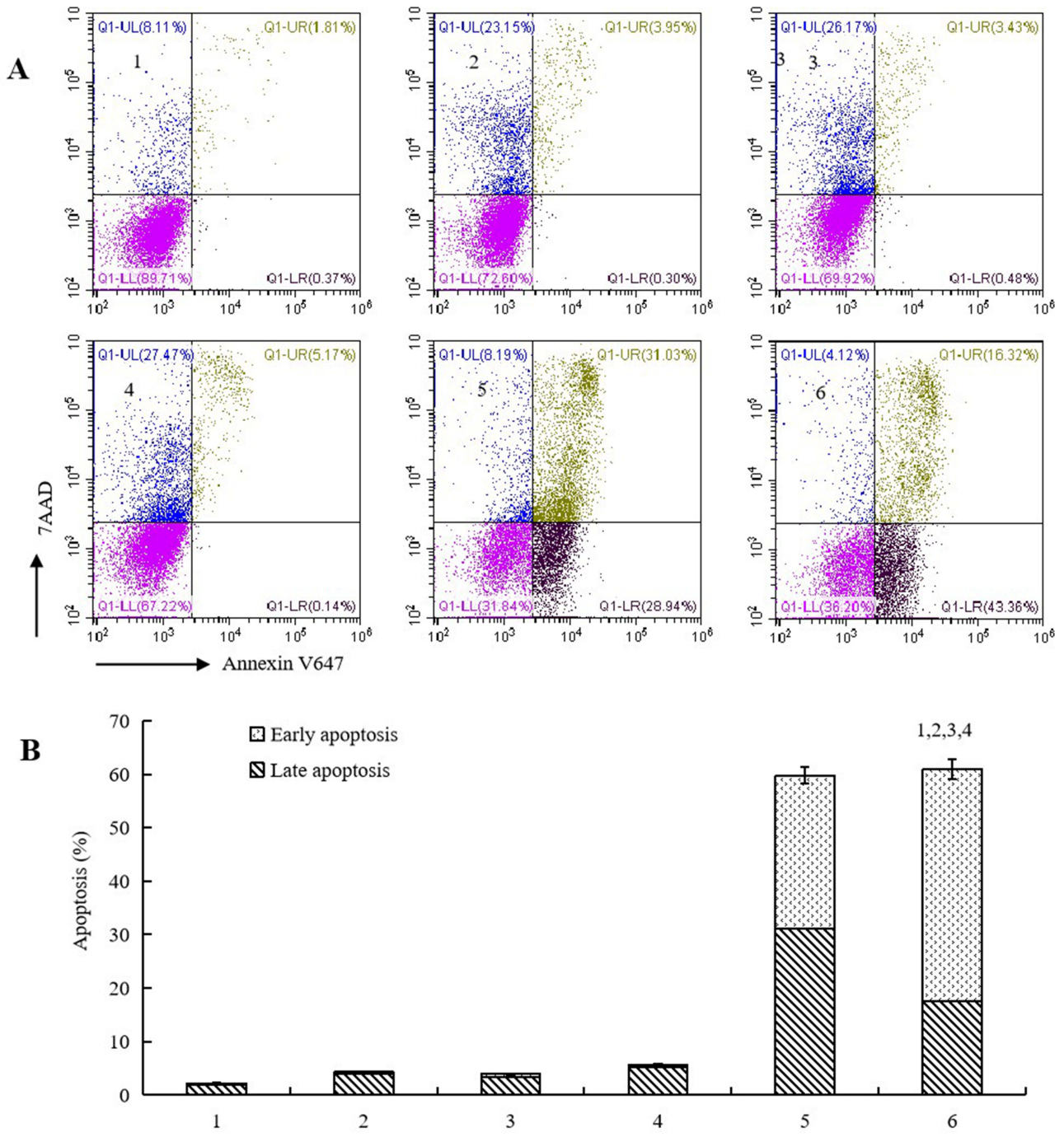


Figure 7 Induced apoptosis on MCF-7/Adr cells after treatment with various formulations.
Notes: (**A** and **B**) 1, control; 2, pure DTX (10 μM); 3, pure (DTX+CUR) (5 μM for DTX; 5 μM for CUR); 4, DTX-loaded-micelles (10 μM); 5, DTX-loaded mixed micelles (10 μM); 6, (DTX+CUR)-loaded mixed micelles (5 μM for DTX; 5 μM for CUR). P, 0.05. Data are presented as mean ± SD (n = 3).
Abbreviation: 7AAD, 7-aminoactinomycin D.

In vivo Antitumor Activity

Regarding the effective cytotoxicity observed against MCF-7/Adr cells in vitro, (DTX+CUR)-loaded mixed micelles showed promise as potential nanocarriers for the treatment of drug-resistant breast cancer. Consequently, the antitumor effects of these micelles on mice bearing MCF-7/Adr tumors were evaluated by monitoring changes in tumor volume throughout the study. Figure 10A displays the tumor growth changes across

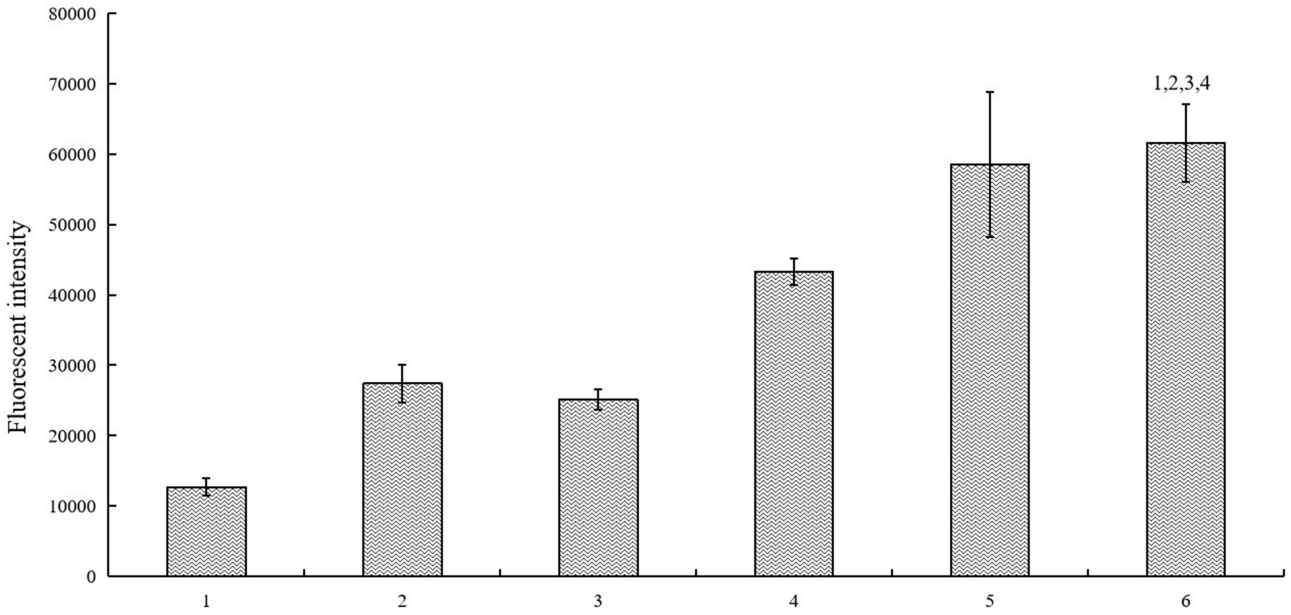


Figure 8 Intracellular ROS on MCF-7/Adr cells after treatment with various formulations.
Notes: 1, control; 2, pure DTX (10 μM); 3, pure (DTX+CUR) (5 μM for DTX; 5 μM for CUR); 4, DTX-loaded-micelles (10 μM); 5, DTX-loaded mixed micelles (10 μM); 6, (DTX+CUR)-loaded mixed micelles (5 μM for DTX; 5 μM for CUR). P, 0.05. Data are presented as mean ± SD (n = 5).

different treatment groups, revealing that (DTX+CUR)-loaded mixed micelles significantly inhibited tumor growth and demonstrated superior therapeutic efficacy.

To assess the safety of the formulations, changes in the body weight of the mice were tracked during the experiment. Figure 10B illustrates the body weight changes among various treatment groups. Notably, the group treated with intravenous injections experienced significant weight loss, whereas mice orally administered with (DTX+CUR)-loaded mixed micelles gained weight, indicating the safety of this formulation. These findings suggested that the oral delivery of DTX and CUR via mixed micelles could enhance efficacy, reduce toxicity, and lower drug dosages. Therefore, these results supported the use of (DTX+CUR)-loaded mixed micelles as a viable drug delivery system for treating drug-resistant breast cancer.

Table 2 Pharmacokinetics Parameters of DTX in Rats After Oral Administration (Mean ± SD, n = 3)

Parameters	Pure DTX (20 mg/kg for DTX)	(DTX+CUR)-Loaded Mixed Micelles (13.74 mg/kg for DTX, 6.26 mg/kg for CUR)
$T_{1/2}$ (h)	3.27±1.53	44.62±27.81*
C_{max} (ng/mL)	490.34±91.53	632.14±61.09*
$AUC_{0-\infty}$ (h.ng/mL)	5402.21±2073.39	31,029.41±367.69*
T_{max} (h)	1.83 ± 0.45	2.11±0.67
MRT (h)	12.72±4.71	67.24±38.00*
F_{rel}(%)	-	574

Abbreviations: Tmax, peak concentration time; Cmax, peak concentration; AUC, area under the curve; T1/2, plasma half-time; MRT, mean retention time; DTX, docetaxel; CUR, curcumin; Frel, relative bioavailability; h, hours. *p <0.05.

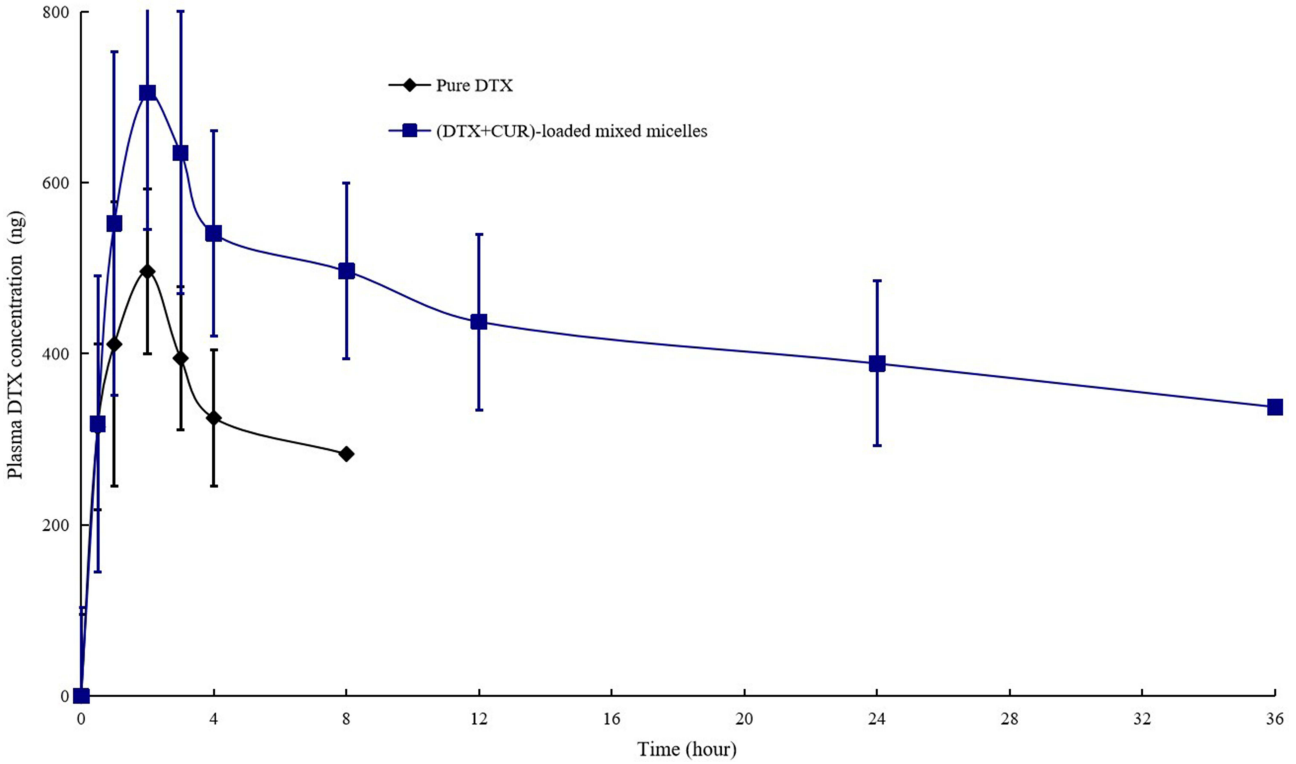


Figure 9 Mean plasma concentration-time curves of DTX in rats after oral administration.
Notes: Pure DTX (20 mg/Kg, p.o.) and (DTX+CUR)-loaded mixed micelles (13.74 mg/kg for DTX and 6.26 mg/kg for CUR, p.o.). Data are presented as mean ± SD (n = 6).

Discussion

Chemotherapy is among the most effective methods for treating cancers. However, the effectiveness of chemotherapy, particularly when using a single drug, often faces challenges such as low bioavailability, the development of drug resistance, and adverse side effects. Employing a co-delivery system that integrates multiple drugs with distinct physicochemical properties and therapeutic effects offers a viable approach to mitigating these issues. Such a strategy not only aims to counteract the above mentioned drawbacks but also seeks to achieve a synergistic effect, thereby enhancing treatment outcomes in cancer therapy.[27] The exploration of well-organised drug delivery systems that encapsulate multiple drugs in a singular formulation is crucial. Over recent decades, there has been a marked increase in research efforts dedicated to developing drug systems designed for co-delivery, utilizing micelles, liposomes, and nanoparticles.[28–30] This approach has led to significant improvements, including enhanced oral bioavailability, greater anti-tumor efficacy, and the reversal of multidrug resistance (MDR), as evidenced by various studies.[31,32]

In this study, we developed a combination therapy using CUR and DTX in mixed micelles for the oral treatment of MDR breast cancers. The suitability of these (DTX+CUR)-loaded mixed micelles for oral administration stemmed from their nanostructured form and the action of the functional material TPGS. These micelles were characterized by their nanosize with a narrow distribution, high drug loading and encapsulation efficiency, and slow release properties. The optimal particle size of 50–100 nm enhanced oral absorption by facilitating transport across the gastrointestinal tract and into the bloodstream.[33] Moreover, the pegylated TPGS aided in evading rapid uptake by the reticuloendothelial system (RES), while the nanostructure increased the permeation and retention (EPR) effect, effectively directing the nanomicelles to the tumor site.[20] Additionally, soluplus could form hydrogen bonds with the drugs (CUR and DTX), which improved the stability of the nanomicelles, increased drug loading and encapsulation efficiency, and minimized drug leakage.[19]

Our findings revealed that CUR markedly boosted the anticancer effects of DTX on MCF-7/Adr cells, while it did not affect the cytotoxicity of DTX on MCF-7 cells. CUR treatment alone had no impact on either MCF-7 or MCF-7/Adr cells

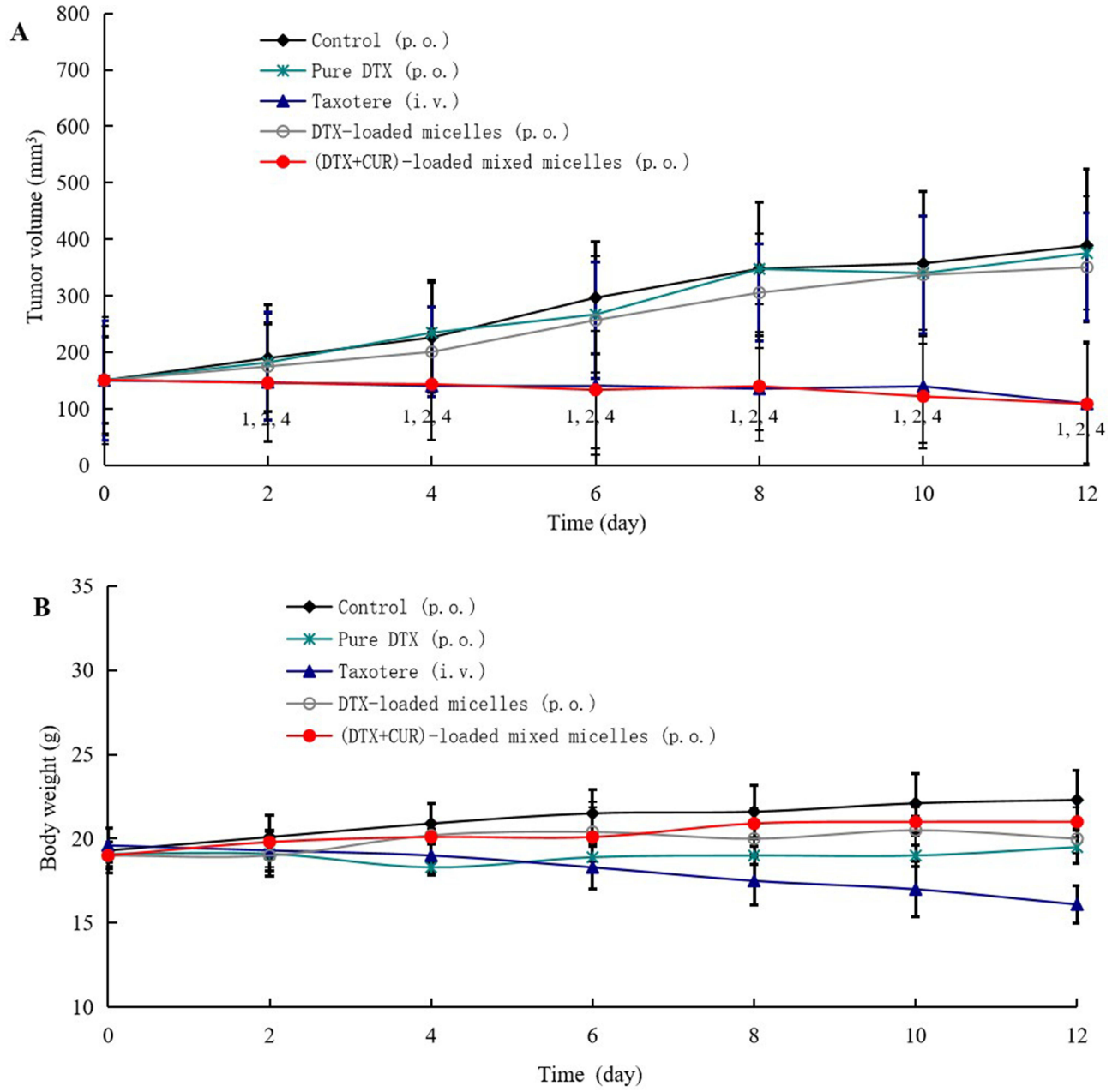


Figure 10 Antitumor efficacy by oral administration of (DTX+CUR)-loaded mixed micelles in drug resistant breast cancer-bearing mice.
Notes: (**A**) Tumor volume changes and (**B**) body weight changes of mice after administration. 1, control (saline orally, p.o.); 2, pure DTX (10 mg/Kg, p.o.); 3, taxotere (10 mg/Kg, i.v.); 4, DTX-loaded micelles (10 mg/Kg, p.o.); 5, (DTX+CUR)-loaded mixed micelles (6.87 mg/Kg for DTX and 3.13 mg/Kg for CUR, p.o.). P, 0.05; Data are presented as mean ± SD (n = 6).
Abbreviations: p.o., orally; i.v., intravenous.

at the concentrations tested. Furthermore, several studies have recognized CUR as an inhibitor of MDR transporters.[34,35] CUR's ability to inhibit P-gp and reduce the efflux of chemotherapeutic agents, which are substrates of MDR transporters, has been documented. Due to CUR's inhibitory effect on Pgp, it could increase the intracellular accumulation of DTX in MCF-7/Adr cells, thereby reversing drug resistance and enhancing cytotoxicity.

To further investigate how CUR overcomes MDR, we assessed its impact on the intracellular accumulation of DTX in drug-resistant MCF-7/Adr cells using fluorescence microscopy and flow cytometry. When treated with free Rh123 alone, MCF-7/Adr cells exhibited a low level of intracellular Rh123 accumulation. This minimal intracellular drug concentration was a key factor in the resistance of MCF-7/Adr cells to DTX monotherapy. The co-encapsulation of CUR and

Rh123 in micelles significantly enhanced the intracellular accumulation of Rh123 in MCF-7/Adr cells, aligning with literature reports that CUR inhibits P-gp mediated drug efflux.[36] In essence, CUR counteracted MDR by directly inhibiting the activities of MDR transporters, thereby diminishing drug efflux and increasing the intracellular concentration of Rh123 in MCF-7/Adr cells. Additionally, the increased cellular uptake of the (DTX+CUR)-loaded mixed micelles might also be attributed to the inhibited drug efflux by ABC transporters, facilitated by TPGS.

The (DTX+CUR)-loaded mixed micelles demonstrated the most potent apoptosis-inducing effect on MCF-7/Adr cells among all tested formulations. A high intracellular concentration of DTX was crucial for inducing apoptosis, making the apoptosis-inducing capability of the dual-drug mixed micelles superior to that of pure DTX. Furthermore, the rate at which DTX is released from the mixed micelles might limit the rate of cellular apoptosis. Unlike free DTX, which was quickly released and eliminated upon entering cells, DTX from mixed micelles was released more slowly, maintaining high concentrations for longer durations. In the dual-drug mixed micelles group, DTX was efficiently transported into cells aided by TPGS, which could inhibit P-gp.[20] Additionally, CUR also inhibited P-gp, preventing the efflux of DTX from cells.[37] This effective transport ensured high intracellular DTX concentrations and maximized the number of apoptotic cells.

The enhanced relative oral bioavailability illuminated the significant benefits of mixed micelles in boosting the therapeutic efficacy of DTX. The oral efficacy of (DTX+CUR)-loaded mixed micelles was shown to be comparable to the intravenous injection of taxotere, demonstrating its potential in inhibiting resistant breast cancer. The results indicated that DTX-loaded micelles alone could only partially counteract MDR, but their effectiveness was significantly enhanced when combined with CUR. Incorporating CUR with DTX in mixed micelles, alongside TPGS, ensured concurrent delivery to the same sites while mitigating toxic side effects, highlighting the formulation's potential for further development. The therapeutic efficacy of the (DTX+CUR)-loaded mixed micelles could be attributed to: (1) A significant increase in the solubility of DTX, from 2.903 μg/mL to 5130 μg/mL.[6] (2) The particle size was crucial for the rate of oral absorption of nanoparticles; specifically, nanomicelles measuring ~64 nm were efficiently absorbed in the gastrointestinal tract and reduced uptake by the mononuclear phagocyte system, thereby extending the drugs' retention time in the blood circulation.[38] (3) Mixed micelles could be entirely absorbed by intestinal epithelia.[39] (4) The role of TPGS as an excipient inhibited intestinal P-gp drug efflux, thereby enhancing the oral absorption of anticancer drugs and delivering more drugs into cells by inhibiting P-gp in MCF-7/Adr cells.[40] (5) CUR's critical role in combating drug-resistant breast cancer by preventing the P-gp-mediated efflux of DTX from MCF-7/Adr cells, consistent with previous studies.[35] However, the precise mechanism was yet to be fully understood and remained a key focus of our future research.

Conclusion

In this study, we successfully prepared (DTX+CUR)-loaded mixed micelles for co-delivery, achieving a synergistic effect on MCF-7/Adr cells between DTX and CUR. These mixed micelles exhibited favorable physicochemical properties, including a uniform spherical shape and an optimal particle size of approximately 64 nm, making them suitable for passive targeting. The in vitro release profile showed sustained drug release characteristics for the (DTX+CUR)-loaded mixed micelles. These micelles demonstrated enhanced cellular uptake, cytotoxicity, apoptosis rate, and reactive oxygen species levels. The co-delivery system notably extended the duration of effective drug concentration in plasma and improved oral bioavailability. This micellar structure presented significant advantages in nanomedicine design: (1) It enabled the co-delivery of hydrophilic drugs, which could be conjugated to TPGS, and hydrophobic drugs, encapsulated within the micellar core. (2) It facilitated a synergistic interaction between the chemosensitizer and the anticancer drug by overcoming multidrug resistance in cancer cells. (3) The co-delivery system enhanced therapeutic effects while minimizing side effects. These findings suggested that the simultaneous delivery of DTX and CUR via mixed micelles could be a promising approach for treating drug-resistant breast cancer.

Acknowledgments

The support from the Guangdong Basic and Applied Basic Research Foundation (2022A1515140129), the Medical Scientific Research Foundation of Guangdong Province (A2023253), and Youth Cultivation Fund of Guangdong Medical

University (GDMUQ2022005), China, is gratefully acknowledged. Their contributions have been pivotal in advancing this research.

Disclosure

The authors report no conflicts of interest in this work.

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