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RESEARCH PAPER

Novel Mixed Polymeric Micelles for Enhancing Delivery of Anticancer Drug and Overcoming Multidrug Resistance in Tumor Cell Lines Simultaneously

Xinru Li • Pingzhu Li • Yanhui Zhang • Yanxia Zhou • Xingwei Chen • Yanqing Huang • Yan Liu

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ABSTRACT

Purpose To evaluate novel mixed polymeric micelles based on monomethoxy poly(ethylene glycol)-poly(D,L-lactic acid) (mPEG-PLA) and Pluronic L61 for delivery of paclitaxel (PTX) to circumvent unfavorable effects resulting from Cremophore EL in Cremophore EL-based PTX formulation and overcoming multidrug resistance (MDR) in tumor cells at the same time.

Methods PTX-loaded plain micelles and mixed micelles were prepared and characterized by determining PTX release *in vitro*, MDR reversal effect in human breast cancer MDR MCF-7/ADR cell sublines and pharmacokinetics *in vivo*.

Results Both PTX-loaded plain micelles and mixed micelles had similar *in vitro* release profile. Mixed micellar PTX significantly reduced IC_{50} of PTX in MCF-7/ADR cells compared to free PTX and plain micellar PTX, and mixed micelles substantially enhanced cellular accumulation of R 123 in MCF-7/ADR cells compared to free R123 and plain micelles. PTX-loaded mixed micelles with lower content of L61 exhibited comparable cytotoxicity to that observed with Cremophore EL-based PTX formulation in inhibiting the growth of MCF-7/ADR cells. Moreover, plain micelles and mixed micelles retained the pharmacokinetic characteristics of PTX in rats compared with Cremophore EL-based PTX formulation.

Conclusion This study suggested that the mixed micelles could enhance delivery of PTX and cell-killing effect for MDR MCF-7/ADR cells.

KEY WORDS methoxy poly(ethylene glycol)-poly(D,L-lactic acid) (mPEG-PLA) · mixed polymeric micelles · multidrug resistance (MDR) · paclitaxel · pluronic L61

INTRODUCTION

The resistance of cancer cells to multiple structurally unrelated chemotherapeutic drugs termed "multidrug resistance (MDR)" has been recognized as a major obstacle to the success of cancer chemotherapy (1). It is well known that overexpression of drug efflux transporters, stress-response proteins, and anti-apoptotic factors in tumor cells results in the development of drug resistance (2–4). Of these mechanisms, overexpression of P-glycoprotein (P-gp), a drug efflux transporter, is the most commonly encountered in the clinic (5). P-gp is an ABC transporter and a plasma membrane glycoprotein encoded by the human MDR1 gene, and it acts as a drug efflux pump that extrudes a wide range of structurally and mechanistically different chemotherapeutic drugs out of cancer cells (6,7). Nearly 40–50% of the patients diagnosed with cancer have P-gp overexpression in the malignant tissue (8). Hence, a common strategy to circumvent P-gp-based MDR is to co-administer a P-gp inhibitor along with the anticancer drug. In general, efflux pump inhibitors can be divided in small molecule inhibitors (SMIs) and polymeric inhibitors. First-generation SMIs (e.g., quinidine, cyclosporine A, verapamil) were limited by unacceptable toxicity (9,10), whereas second-generation SMIs (e.g., PSC833, VX-710) had

Xinru Li and Pingzhu Li contributed equally to this work.

X. Li • P. Li • Y. Zhang • Y. Zhou • X. Chen • Y. Liu (✉)
Department of Pharmaceutics, School of Pharmaceutical Sciences
Peking University
Beijing 100191, China
e-mail: yanliu@bjmu.edu.cn

Y. Huang
Pharmaceutical Teaching Experiment Center
School of Pharmaceutical Sciences, Peking University
Beijing 100191, China

better tolerability but were limited by unpredictable pharmacokinetic interactions with the anticancer drug and interactions with other transport proteins (6,11). Third-generation SMIs (e.g., tariquidar, zosuquidar, laniquidar, and ONT-093) have high potency and specificity for P-gp. However, as of today, most SMIs of P-gp have failed during pre-clinical or clinical development because of low efficiency and substantial side effects (12,13). Recently, some widely used and so-far-believed pharmacological inactive polymeric pharmaceutical excipients including Pluronics® have been identified to inhibit efflux pumps. Pluronics®, amphiphilic synthetic polymers containing hydrophilic poly(ethylene oxide) (PEO) blocks and hydrophobic poly(propylene oxide) (PPO) blocks arranged in a triblock structure PEO-PPO-PEO (14), were shown to enhance the drug uptake and reduce the P-gp-mediated drug efflux (15,16), especially for L61 (17) with molecular weight of 2,000 Da and the average numbers of EO and PO units of 4.5 and 31, and P85(15,18,19). The efflux pump inhibitory mechanism is believed to be due to (a) Pluronic-mediated inhibition of P-gp ATPase activity and ATP depletion (20) and (b) effect of Pluronics on membrane fluidization, and it has been proved that the effect of Pluronics on energy conservation is mediated by single-chain units (unimers), and the inhibitory effect decreases when reaching the critical micelle concentration (21). Notably, an important concern is that the difference in physico-chemical properties of the anticancer drug with efflux inhibitor may result in differences in the pharmacokinetics and accumulation of the two agents in tumor site. Both the drug and the inhibitor may need to be temporally colocalized in the tumor cells for optimal synergy. This can be achieved only by using a delivery system co-encapsulating the two agents.

Several studies over the past decade have extensively documented the advantages of polymeric micelles as anticancer drug carriers, such as their ability to overcome limitations stemming from toxicity, solubilize poorly water-soluble drugs and prolong blood circulation time (22,23). Polymeric micelles are nanoscopic (<100 nm) vehicles self-assembled by amphiphilic block copolymers with hydrophilic and hydrophobic chains in water above a certain concentration named the critical micelle concentration (CMC) (22). Micelles comprise an inner and outer domain-denominated core and shell, respectively. Due to their core-shell architecture, they can reduce side effects of anticancer drugs, protect unstable drugs from chemical degradation and metabolism by biological agents (24). Polymeric micelles are safer for parenteral administration than solubilizing agents currently in use, like polyethoxylated castor oil (Cremophore EL) or polysorbate 80 (Tween 80) (25–27) and kinetically stable even at concentrations below the CMC (27). In addition, they display larger cores than surfactant micelles, leading to higher solubilization capacity than the regular micelles (27). Micelles with blocks made of poly(ethylene oxide) are sterically stabilized and undergo less opsonization and uptake by the macrophages of the reticuloendothelial system (RES), allowing the micelles to circulate longer in blood (27,28). Moreover, polymeric micelles have the right size to avoid renal excretion but allow extravasation at the tumor site. This provides for passive tumor targeting via the enhanced penetration and retention (EPR) effect. Recently, polymeric micelles have been evaluated in several clinical trials as carriers for anticancer drugs (29–31). Zhang *et al.* reported on the antitumor activity and biodistribution of paclitaxel (PTX) loaded in polymeric micelles based on PEO-*b*-poly (DL-lactide) (32). They demonstrated that both polymeric micellar PTX and Cremophore EL-based PTX exhibited comparable efficacy in inhibiting the growth of Hs578T breast tumor cells, SK MES non-small cell lung tumor cells, and HT-29 colon tumor cells. Our previous work also demonstrated the beneficial effect of lactobionic acid-functionalized Pluronic P105 micelles in liver-targeted delivery of silybin (33). Moreover, it was reported that diblock copolymer PEGylated polyesters alone could not effectively suppress the P-gp drug efflux function regardless of whether it was in unimer or micellar form (34). Dual functions of solubilization as well as P-gp inhibition were hence expected for Pluronic block copolymers. Wang and coworkers designed difunctional Pluronic copolymer micelles for paclitaxel delivery and Pluronic-mediated overcoming multidrug resistance in tumor cell lines (35). They reported that the MDR cells were more susceptible to the cytotoxic effects of Pluronic micellar paclitaxel than their parental cells. However, the cytotoxicity of paclitaxel-loaded P105 micelles or P105/L101 mixed micelles was not compared with that of Cremophor EL-based paclitaxel formulation widely used in clinic, only compared with that of free paclitaxel in the cytotoxicity experiments. Furthermore, the more hydrophilic representatives of Pluronic family of compounds display lower micellization ability (with higher CMC) at 25°C, and their micelles had lower solubilization capacity (with considerably lower loading content) (35,36). Next, Pluronic block copolymers are non-biodegradable. These are major obstacles for Pluronic micelles to be used as drug carriers. Consequently, these previous results prompted us to overcome these drawbacks by introducing a Pluronic compound into a polymeric micelle with lower CMC and higher solubilization capacity, which formed mixed polymeric micelle. This technique would improve the hydrosolubility of the drug and, importantly, overcome two main complications of cancer chemotherapy: severe side effects of toxic drugs and resistance of cancerous cells to drug action, which is either inherent or developed in the course of chemotherapy.

Paclitaxel (PTX) is one of the most important anticancer agents for a broad spectrum of human malignancies. Due to a poor aqueous solubility of approximately 1 μg/ml, PTX is currently formulated in 1:1 (v/v) Cremophor EL and dehydrated ethanol mixture. This formulation has some serious associated side effects, including hypersensitivity, neurotoxicity and nephrotoxicity, and leads to extraction of plasticizers from intravenous infusion line and to precipitation on aqueous dilution, which is believed to be caused by the Cremophor EL (37–39). Another significant obstacle for success of chemotherapy with PTX is multidrug resistance (MDR) in many cancer cells (40). Recently, a commercially available PTX formulation in PEG2000-PLA1750 polymeric micelles called Genexol-PM was reported. According to the literature (34) and our preliminary test, diblock copolymer PEGylated polyesters alone could not effectively suppress the P-gp drug efflux function regardless of whether it was in unimer or micellar form.

The purpose of the present work was to propose a novel polymeric micelle platform for delivery of anticancer drug and overcoming drug resistance in cancer cells at the same time. PTX-loaded polymeric micelles have been reported previously (41,42). We proposed mixed micelles fabricated with monomethoxy poly(ethylene glycol)-poly(D,L-lactic acid) (mPEG-PLA) and Pluronic L61. Therefore, the synthesis of mPEG5000-PLA3250 was presented. The micelles preparation, PTX solubilization and micelles properties were investigated by the size measurement, drug loading content, drug loading efficiency and *in vitro* drug release. The influence of L61 on CMC of mPEG5000-PLA3250 was evaluated. The effects of mixed micelles on the cytotoxicity of PTX to MCF-7/ADR breast cancer cells and the uptake of Rhodamine (R123) by MCF-7/ADR cells were also evaluated *in vitro* as compared with those of Cremophor EL-based PTX commercial formulation, free drug and plain micelles, respectively. Moreover, pharmacokinetics characteristic of PTX-loaded plain micelles and mixed micelles after intravenous administration to rats were assessed in comparison to Cremophor EL-based PTX commercial formulation.

MATERIALS AND METHODS

Materials

Monomethoxy poly (ethylene glycol) (mPEG) with M_W 5000 was obtained from Merck (USA). D,L-lactide was purchased from Fushun Tianyuan Biomaterial Co. Ltd. (Fushun, China) and recrystallized three times in ethyl acetate before use. Pluronic L61 was supplied by BASF (Parispany, NJ, USA). Stannous 2-ethylhexanoate was purchased from Sigma-Aldrich (Milwaukee, WI, USA). Paclitaxel was purchased from Guilin Huiang Biopharmaceutical Co. Ltd. (Guilin, China). Tetrazolium salt MTT and trypsin were obtained from Sigma-Aldrich. (St. Louis, MO, USA). R123 was purchased from Sigma (USA). RPMI-1640 medium and DMEM high glucose medium were purchased from Tianrunshanda Biotech Co. Ltd. (Beijing, China). Fetal bovine serum (FBS) was provided by Biodee Co. Ltd (Beijing, China). Human breast cancer MCF-7 cells were provided by Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences (Beijing, China), and their MDR MCF-7/ADR cell sublines were derived from the parental cells by selection with Dox, i.e. created by selecting live MCF-7 cells following stepwise exposure to free Dox at 0.001–10 mg/ml in our own lab. Kits for determination of protein concentration were purchased from Bio Cellchip Co. Ltd. (Beijing, China).

Animals

Sprague-Dawley rats weighing 200 ± 20 g were obtained from Experimental Animal Center of Peking University and maintained on a light/dark cycle. All animals were allowed free access to standard rat chow and water. Temperature and relative humidity were maintained at 25°C and 50%, respectively. Rats were acclimatized for 7 days prior to experiments. All care and handling of animals were performed with the approval of Institutional Authority for Laboratory Animal Care of Peking University.

Synthesis and Characterization of mPEG-PLA

mPEG5000-PLA3250 diblock copolymer was synthesized by a ring-opening polymerization procedure as described previously (41,43) with little modification. In brief, the starting materials (5 g of mPEG and 3 g of D,L-dilactide) were each dissolved in 100 ml toluene in a round-bottomed flask. After about 40 ml of toluene was distilled off, the two solutions were mixed in a three-neck flask followed by addition of 15 mg of stannous 2-ethylhexanoate. The reaction mixture was then refluxed for 24 h at 140°C under nitrogen. After toluene was distilled off, the residue was redissolved in appropriate amount of dichloromethane. The precipitation was performed by addition of ten-fold ice-cold diethyl ether under vigorous stirring. A white powder was sequentially isolated by filtration and dried under high vacuum. The resulting copolymer was dissolved in $CDCl_3$, and ^{1}H-NMR spectra were taken at 300 MHz with trimethylsilane (TMS) as internal reference standard using a Bruker MSL2300 spectrometer (Bruker, Germany).

Determination of Critical Micelle Concentration

The critical micelle concentration (CMC) of mPEG-PLA diblock copolymers was determined by fluorescence measurements using pyrene as a hydrophobic probe as previously reported (44). The fluorescence spectra of pyrene were measured at varying polymer concentrations using a Shimadzu RF-5301 PC fluorescence spectrophotometer at 25°C. The excitation wavelength was adjusted to 392 nm, and the detection of fluorescence was performed at 333 nm and 335 nm. CMC was measured from the onset of a rise in the intensity ratio of peaks at 335 nm to peaks at 333 nm in the fluorescence spectra of pyrene plotted *versus* the logarithm of polymer concentration.

Preparation of PTX-Loaded Polymeric Micelles

PTX-loaded polymeric micelles were prepared by a rotary evaporation method (42). Briefly, 1.0 mg of PTX and 10 mg of mPEG-PLA were co-dissolved in 5 ml of acetonitrile. After the mixture was sonicated for 5 min, the organic solvent was evaporated under vacuum at 60°C with a rotary evaporator to obtain a transparent gel matrix. Then, 10 ml of phosphate buffer solution (PBS, pH 7.4) was added at 60°C and vortexed for 5 min. The suspension was then filtered through a 0.22 μm membrane filter to remove aggregates, and a transparent PTX-loaded micelle (plain micelle) solution was obtained. Mixed polymeric micelle was prepared as described above, except mPEG-PLA was replaced by mPEG-PLA and L61. The lyophilized powder of PTX-loaded micelle was obtained by lyophilization of micelle solutions with a freeze-dryer system (Sihuan LGL0.5, Beijing, China).

Characterization of Micelles

Determination of Micelle Size and Size Distribution

Micelle size and size distribution, in terms of polydispersity index (PDI), were determined by using dynamic light scattering (DLS) method (Malvern Zetasizer ZEN3500, UK). All measurements were performed at a wavelength of 532 nm with a scattering angle of 173° at 25°C after diluting the dispersion to an appropriate volume with PBS.

Determination of Drug Loading Content and Loading Efficiency

To determine drug loading content (LC, w/w%) and loading efficiency (LE, w/w%) of micelles, the lyophilized powder of PTX-loaded micelle was dissolved in acetonitrile by ultrasonication for 15 min, and PTX content was measured by HPLC at the wavelength of 227 nm. The solution was properly diluted prior to HPLC analysis. The HPLC system (Shimadzu LC-10AT, Kyoto, Japan) was equipped with a UV detector (Shimadzu SPD-10A) and reversed phase column (Diamonsil C-18, 4.6 mm × 250 mm, Dikma Technologies, China). The mobile phase was composed of acetonitrile:methanol:H_2O (37.5:37.5:25). The mobile phase was pumped at a flow rate of 1.0 ml/min. The column temperature was set to 30°C. The LC and LE were then calculated using the following equation: LC% = (weight of PTX extracted from lyophilized micelles/weight of lyophilized micelles) × 100%; LE% = (weight of PTX extracted from lyophilized micelles/weight of feed PTX) × 100%.

Evaluation of Mixed Polymeric Micelle Stability in Physiological Condition

The stability of mixed polymeric micelles in physiological condition was evaluated by determination of leakage percent of PTX from mixed micelles in rat plasma. Briefly, the rat plasma was collected by centrifugation and then mixed with the mixed micelle solution in a vial in the volume ratio of 1:1. The vial was placed in a water bath shaking at 100 rpm at 37°C. At the designated time intervals, 0.5 ml of the mixture in the vial was sampled and ultrafiltered using an ultrafilter (Millipore, USA) with molecular weight cutoff of 100,000. The ultrafiltrate containing free PTX and plasma protein-bound PTX was extracted and analyzed by HPLC method as described in the Pharmacokinetics Evaluation section. The leakage content of PTX from mixed micelles in plasma was calculated. All experiments were carried out in triplicate.

In Vitro Release of PTX from Micelles

In vitro release of PTX from micelles was performed against a sink medium (1 mol/l sodium salicylate solution) using the membrane diffusion technique (45). One milliliter of micelle solution (the content of the drug was 0.11 mg, 0.10 mg, 0.094 mg and 0.078 mg for plain micelles, mixed micelles with 9.09%, 16.7% and 28.6% L61, respectively) obtained by reconstituting freeze-dried micelles in PBS was introduced into a dialysis bag (MWCO 3500) and then immersed into 40 ml release medium in an orbital water bath shaking at 100 rpm at 37°C. At the designated time intervals, the release medium outside the dialysis bag was removed and filtered with a 0.22 μm membrane filter for HPLC analysis and replaced with fresh release medium. In the assessment of drug release behavior, the cumulative amount of the released drug was calculated, and the cumulative release percentages of drug released from each micelle were plotted against time.

Cell Culture

Human breast cancer MDR MCF-7/ADR cell sublines, derived from the parental cells by selection with doxorubicin, were maintained in RPMI-1640 culture medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 U/ml) and L-glutamine. The cells were cultured at 37°C in humidified environment with 5% CO_2. The culture condition for MCF-7 cells was similar to that of MCF-7/ADR, but the culture medium was Dulbecco's modified Eagle's medium (DMEM) High Glucose instead. The cells in their logarithmic growth phase were harvested with trypsin for the further experiments. MCF-7 or MCF-7/ADR cells were seeded at a density of 5,400 cells/well in 180 μl culture medium on 96-well plates and allowed to grow overnight for reattachment.

In Vitro Cytotoxicity Assay

Serial dilutions of PTX-loaded micelle solution were performed by adding appropriate volume of PTX-loaded micelle solution to drug-free micelle solution to give a series of PTX concentrations. The content of micelle-forming polymer was 2 mg/ml in each obtained micelle solution. Free PTX solution prepared by dissolving PTX in PBS solution containing 1.0% DMSO as cosolvent was used as a control. Cremphore EL-based commercial formulation, drug-free micelle solution and PBS solution containing 1.0% DMSO were also tested.

The cytotoxicities of tested samples were assessed by a standard thiazolyl blue tetrazolium bromide (MTT) assay as described previously (35). Briefly, MCF-7 and MCF-7/ADR cells were incubated in 96-well plates as described above. After seeding for 24 h, 20 μl of serial dilutions of tested samples were added to each well. Cells were incubated for a further 72 h, and then 20 μl of 5 mg/ml MTT dissolved in PBS was added to each well, and the cells were incubated for additional 4 h. Then the medium was removed completely, and 150 μl of dimethyl sulphoxide (DMSO) was added to each well to dissolve purple formazan crystal. The number of viable cells in each well was then determined by absorbance at the wavelength of 540 nm measured on a microplate reader (Model 680, BIO-RAD Laboratories, Japan), and obtained values were expressed as a percentage of the values obtained for control cells to which no carriers were added. These values were then expressed as survival percentage, and IC_{50} values were calculated using nonlinear regression analysis. All the experiments were carried out in sextuplicates. The MDR reversal effect was assessed by quantifying the IC_{50} values of PTX for all the tested formulations.

Cellular Accumulation of Rhodamine 123 (R123)

R123 accumulation in cells was studied as previously described (46). Briefly, solutions of R123, R123-loaded plain micelles and mixed micelles with 1.96% and 3.85% L61 were prepared in culture medium without FBS. MCF-7/ADR cell monolayers were preincubated in 24-well plates for 30 min at 37°C in culture medium with 10% FBS. After this, the medium was removed, and 5 μmol/l free R123, plain micellar R123 with 5 μmol/l R123, mixed micellar R123 with 5 μmol/l R123 or 5 μmol/l free R123 plus 20 μmol/l cyclosporine A were added to the cells. The cells were incubated with dye solutions for up to 4 h at 37°C. After that, the dye solutions were removed, and cell monolayers were washed three times with ice-cold PBS. The cells were then solubilized in 1.0% Triton X-100, and 25 μl of aliquot was removed for determination of cellular dyes using a Shimadzu RF-5301 PC fluorescence spectrophotometer at $\lambda_{ex}=485$ nm and $\lambda_{em}=530$ nm. Samples were taken for protein assay using BCA method. All experiments were carried out in triplicate.

Pharmacokinetics Evaluation

Fifteen male Sprague-Dawley rats weighing 200 ± 20 g, fasted for 12 h prior to the experiments but allowed free access to water, were divided into three groups. The rats in each group received a single intravenous injection of commercial formulation, plain micelles (0.263 mg/ml of PTX) and mixed micelle (0.254 mg/ml of PTX) with 3.85% (w/w) L61 through the tail vein at an equivalent PTX dose of 5 mg/kg, respectively. 0.7 ml of blood samples were taken through the tail vein at predetermined time intervals after drug administration. The plasma was collected by centrifugation and stored at −20°C until analysis.

Blood Sample Analysis

Liquid-liquid extraction procedure for plasma samples was as follows: in a 10 ml polypropylene screw-capped conical tube was added 300 μl of plasma followed by 100 μl of internal standard solution (70% of α-naphthol in acetonitrile solution), 100 μl of 70% acetonitrile and 2.5 ml of tert-butyl ether. After vigorous vortex mixing for 5 min, an organic layer was collected by centrifugation at 4,000 rpm for 10 min and then transferred into another tube and evaporated under a light stream of nitrogen. The residue was redissolved in 100 μl of 70% acetonitrile. After centrifugation, 20 μl of supernatant was injected for HPLC analysis.

A modified HPLC/UV method was employed to determine PTX level in rat plasma. The Shimadzu series

HPLC system (Shimadzu LC-10AT, Kyoto, Japan) was equipped with a UV detector (Shimadzu SPD-10A) and reversed phase column (ODS C18, 5 μm, 4.6 mm× 250 mm, Dikma, China) guarded with a refillable pre-column (ODS C18, 2.0 mm×20 mm, Alltech, USA). The UV absorbance was detected at 227 nm. The mobile phase was composed of methanol:H_2O:tetrahydrofuran (67.5:30:2.5). The mobile phase was pumped at a flow rate of 1.0 ml/min. The column temperature was set to 30°C. The coefficient of variation of the inter-day and intra-day precision of the quality control samples ranged from 2.27 to 5.23%, and accuracy ranged from 92.1 to 94.5%. The limit of detection (LOD) and the limit of quantification (LOQ) were 14.6 ng/ml and 58.5 ng/ml, respectively. The extraction recovery was higher than 85%.

The pharmacokinetic parameters, including area under the drug concentration-time curve up to last time (AUC), mean residence time up to last time (MRT) and total clearance (CL), were assessed by WinNonLin (Pharsight, Mountain View, CA, USA) using non-compartmental methods.

Statistical Analysis

All the data are presented as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was adopted to determine the significance among groups. A value of $P<0.05$ was considered to be significant.

RESULTS

Synthesis and Characterization of mPEG-PLA

mPEG5000-PLA3250 was synthesized by ring-opening polymerization of D,L-dilactide by using mPEG as initiator. Fig. 1 showed the ^{1}H-NMR spectrum of mPEG5000-PLA3250. The peaks at 3.65 ppm corresponded to methylene units in the mPEG blocks; signals at 1.5 and 5.1 ppm could be attributed to the hydrogen atoms of CH_3- and CH-groups for PLA segments, respectively. From the peak integration ratio of their methylene and methyl groups, the mass ratio of repeating units in mPEG and PLA block can be calculated in a polymer molecule.

Polymeric micelles can be formed only when the block copolymer concentration is higher than CMC, which characterizes the micelle stability (47). One main concern following the use of polymeric micelles for drug carriers is the severe dilution they undergo in the biological environment (sometimes below the CMC). Therefore, the CMC is an effective parameter of micelle preparation. Table I shows the results of influence of L61 content on the CMC of mPEG-PLA. It was found that the CMC was 1.951 mg/l at 25°C for the synthesized mPEG-PLA diblock copolymer and increased slightly with the increase of L61 content, which might be attributed to the reduction of hydrophobic interaction between the hydrophobic segments. It is well known that the stronger the hydrophobic interaction between the hydrophobic segments the lower the CMC of micelle-forming material. In the case of our experiment, the hydrophobic segments forming the core of plain micelles and mixed micelles were PLA chains, and PLA and PPO chains, respectively. The hydrophobic interaction between PLA segments was greater than that between PLA segment and PPO moiety of Pluronic L61 molecules due to the high lipophilic character of PLA, and the more the content of L61 the weaker the hydrophobic interaction.

Micelle Properties

Size and size distribution might play a key role in determining the fate of micelles after administration. The micelles were hence characterized by mean size and polydispersity index (PDI). As shown in Table II, micelles with nanoscaled size in diameter and narrow distribution were successfully prepared. More importantly, it was worth noting that the size was in the nanometer size range and may be exploited to achieve efficient tissue penetration to target sites in the body. Moreover, the micelle size appeared to be dependent on both copolymer composition and drug-loading. Similar to the PTX-loaded polymeric micelles reported earlier (45,48), the diameter of drug-loaded micelles was significantly larger than that of drug-free micelles. In addition, insertion of L61 into plain micelles apparently led to the significant increase in particle size ($P<0.001$). These might be attributed to the fact that it is difficult to form compact polymeric micelles for mPEG-PLA in the presence of L61 owing to the weaker interaction between PPO and PLA segments.

In addition, the loading content and loading efficiency of PTX in mixed micelles significantly decreased ($P<0.05$) with increase of L61 content in micelles (Table I), likely due to the fact that longer PLA chain (as compared with PPO chain) brought about more micellar hydrophobic space for the drug to embed in, as well as the hydrophobic interaction between PLA and PTX was stronger than that between PPO and PTX, or the compatibility between the core of plain micelles and PTX molecules was better than that between the core of mixed micelles and PTX molecules (41).

The stability of mixed polymeric micelles is important to understand *in vitro* drug uptake and *in vivo* absorption; thereby, it was evaluated by determination of leakage content of PTX from mixed micelles in rat plasma. Fig. 2 showed the leakage profile of mixed micelles with 3.85% L61, which was representative for mixed micelles. As was

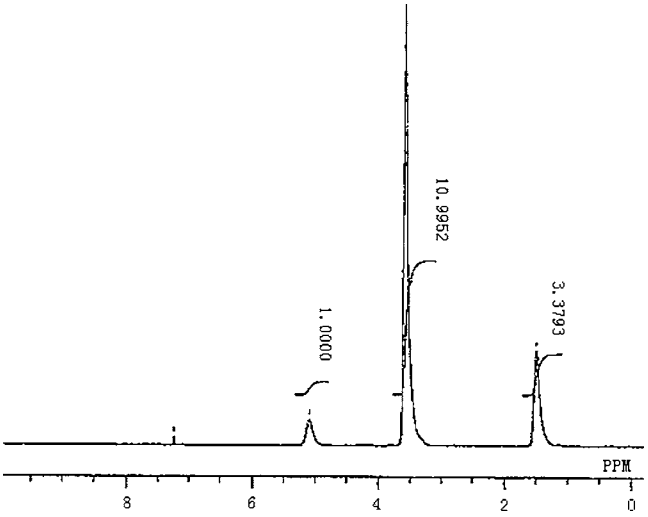

Fig. 1 ^{1}H NMR spectrum of mPEG5000-PLA3250 copolymer in $CDCl_3$.

seen in Fig. 2, there was slow and no significant increase ($P>0.05$) in the leakage percent between the adjacent sampling points from 0.5 h to 2 h. The leakage percent was 14.12±1.89% within 2 h, indicating that the micelles exhibited favorable stability to a certain extent in physiological condition before reaching targeting sites.

In Vitro Release of PTX from Micelles

In general, the cytotoxicity of drug-loading micelles depends on the time of cell exposure to micelles (18). In order to determine the incubation time of MCF-7 and MCF-7/ADR cells with PTX-loaded micelles in *in vitro* cytotoxicity assay, the *in vitro* release properties of PTX from plain micelles and mixed micelles were investigated. PTX is poorly soluble in the cell culture media, so the solubilization agent should be added. Sodium salicylate aqueous solution was selected according to the literature due to the fact that organic solvents such as acetone and DMSO can dissolve micelles. Unfortunately, the precipitate produced after sodium salicylate was added into the cell culture media. Moreover, it was found that the tested micelles were stable in the cell culture media in our preliminary test, and the drug release from micelles was mainly controlled by diffusion. One mol/l sodium salicylate solution was therefore selected to be the release medium in current research, and the sink condition was maintained. Although no exchange of media was performed during the later cytotoxicity test, the sink condition could be achieved due to the fact that the micelle solution (20 μl) was diluted for 10-fold (200 μl) with the culture media in each tested cell. As shown in Fig. 3, the similar release characteristics for plain micelles and mixed micelles were unexpectedly observed, indicating that the insertion of L61 into a mPEG-PLA micellar system and L61 content in mixed micelles did not obviously affect the release behavior of micelles. This finding may be assigned to the factors contributing to drug release from polymeric micelles, such as micelle size, hydrophobic interaction between hydrophobic segments, interaction between the core and the drug molecules (i.e. the compatibility between the core and the drug molecules), drug loading content, and so on. Therefore, the compatibility was not the sole factor influencing drug release from micelles. Briefly, about 80% of PTX was released within the first 4 h, and almost all PTX was released from micelles within 10 h. Moreover, burst release existed for all samples during initial period (Fig. 4).

Table I Effect of L61 Content on the Properties of the Copolymer and Polymeric Micelle at 25°C

L61 content (%, w/w)	CMC (mg/l)	LC (%)	LE (%)
0	1.951	13.37±0.23	93.82±1.76
1.96	2.136	12.75±0.10	89.30±1.39
3.85	2.294	11.42±0.04	75.61±2.41
9.09	2.785	7.76±1.45	56.18±11.24
16.7	3.112	6.99±0.25	50.14±1.92
28.6	3.397	4.67±0.25	32.65±1.82

In Vitro Cytotoxicity of PTX-Loaded Mixed Micelles

To assess *in vitro* cytotoxicity of PTX-loaded mixed micelles to MCF-7 and MCF-7/ADR cells, IC_{50} values were estimated in parental cells and selected cell sublines as compared with free PTX, PTX-loaded plain micelles and Cremophore EL-based PTX formulation. MCF-7 and MCF-7/ADR cells were exposed to serial dilutions of free PTX solution, and PTX concentrations ranged from 0.27 ng/ml to 35 ng/ml and 129.51 ng/ml to 16578 ng/ml, respectively. As one might expect, MCF-7/ADR cells exhibited extremely significant increase in IC_{50} compared to parental cells (Table III), suggesting that the cytotoxicity of free PTX to MCF-7 cells was considerably higher than that to MCF-7/ADR cells, that is to say, the resistant sublines (MCF-7/ADR) demonstrated considerably lower response to the cytotoxic action of free PTX compared to MCF-7 cells. The resistance index, i.e. ratio of IC_{50} of free PTX solution for MCF-7/ADR cells to that for MCF-7 cells, of MCF-7/ADR cells was 1322, implying that MCF-7/ADR cells in current study possessed considerably higher drug resistance to PTX compared to the parental lines. Furthermore, drug-free plain mPEG-PLA micellar solution, mPEG-PLA/L61 mixed micellar solution with L61 content of lower than 9.09% and 1.0% DMSO showed no cytotoxicity against cultured MCF-7 and MCF-7/ADR cells

Table II Properties of Plain Micelle and Mixed Micelle (with 3.85% of L61) (mean ± SD, $n=3$)

	Plain micelle		Mixed micelle	
	Drug-free	Drug-loaded	Drug-free	Drug-loaded
Size (nm)	37.27 ± 1.12	47.79 ± 0.86*	55.60 ± 0.65	72.20 ± 0.53*
PDI	0.27 ± 0.06	0.34 ± 0.01	0.25 ± 0.06	0.31 ± 0.02
LC (%)	–	13.37 ± 0.23	–	11.42 ± 0.04

* $P<0.001$ *vs.* drug-free micelle.

(data not shown). This finding was in accordance with previous studies (35,49).

Fig. 5 showed the inhibition activity for the proliferation of MCF-7/ADR cells by Cremophore EL-based PTX formulation and polymeric micelles at various PTX concentrations. As shown in Table III, the IC_{50} of plain micellar PTX was 6122 ng/ml for MCF-7/ADR cells, which was significantly lower than that of free PTX (11557 ng/ml), and a similar result was observed for MCF-7 cells, indicating that plain micellar PTX had higher cytotoxic activity toward cultured MCF-7 cells and MCF-7/ADR cells compared to that of free PTX. These results were similar to those reported in literature in which Wang *et al.* reported that PTX-loaded P105 micelles could remarkably suppress the P-gp drug efflux function of MCF-7/ADR cells compared to that of free PTX (35). A resistance reversion index (RRI), a ratio of IC_{50} of free PTX solution to that of micellar PTX for MCF-7/ADR (IC_{50} (free PTX)/IC_{50} (micellar PTX)), was commonly used to evaluate the effects of the formulations on the resistant cells. It must be noted that the RRI of plain micellar PTX was 1.89, which was considerably lower than that of P105 micellar PTX (RRI was 14.1), suggesting that the plain micellar formulation in the present study had relatively lower MDR reversal effect.

Our preliminary test found that the drug-free plain micelles and the drug-free mixed micelles with 1.96% and 3.85% L61 had no cytotoxicity to the cells, while the drug-free mixed micelles with 9.09%, 16.7% and 28.6% L61 had cytotoxicity to MCF-7 cells and MCF-7/ADR cells to some extent (data not shown). The mixed micelles with 1.96%, and 3.85% L61 were therefore chosen in the subsequent studies. Excitingly, the enhancement in cytotoxicity of PTX in MCF-7/ADR cells for mixed micellar PTX was observed compared to plain micellar PTX. As shown in Fig. 5 and Table III, the RRI of mixed micelles with 1.96% (0.004 mg/ml) and 3.85% (0.008 mg/ml) of L61 was 6.21 and 11.29, respectively, indicating that mixed micelles had significant MDR reversal effect on MCF-7/ADR cells compared with plain micelles, which presumably resulted from the function of P-gp inhibition of L61 introduced into mixed micelles (17). Additionally, the IC_{50} value of mixed micellar PTX with 3.85% L61 was significantly lower than that of mixed micellar PTX with 1.96% L61($P<0.001$), suggesting that the effect of MDR reversal to MCF-7/ADR cell for mixed micelles was related to the content of L61 in micelles. The higher the content of L61 in mixed micelles, the better the MDR reversal of MCF-7/ADR cells in the tested concentration range of L61.

In addition, it was found that the Cremophore EL-based PTX formulation also had the function of overcoming the MDR as shown in Fig. 5 and Table III, which presumably resulted from the function of P-gp inhibition of Cremophore EL (13). This effect of Cremophore EL seemed to be either due to a direct interaction with P-gp (50,51) or the result of a general membrane perturbation affecting the function of the protein pump (52,53). Cremophore EL at concentrations ≥ 0.1 μl/ml increased the sensitivity of MDR cells, with about 50% reversal at 1.0 μl/ml and complete reversal occurring at concentrations of 1.5–2.0 μl/ml *in vitro* (54,55). The concentration of Cremophore EL in commercial formulation is 0.43 μl/ml. More

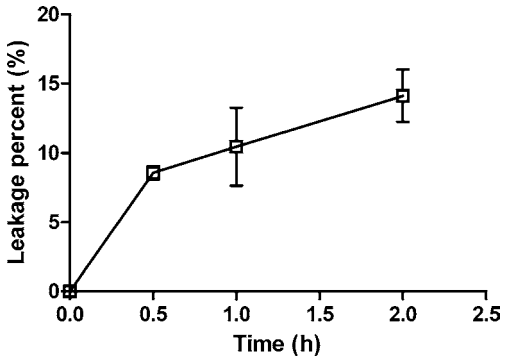

Fig. 2 Leakage profile of mixed micelles with 3.85% L61 in plasma ($n=3$).

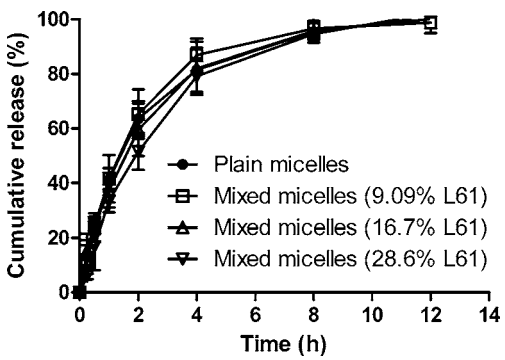

Fig. 3 Release profiles of PTX from plain micelle and mixed micelles in 1 mol/l sodium salicylate solution ($n=4$).

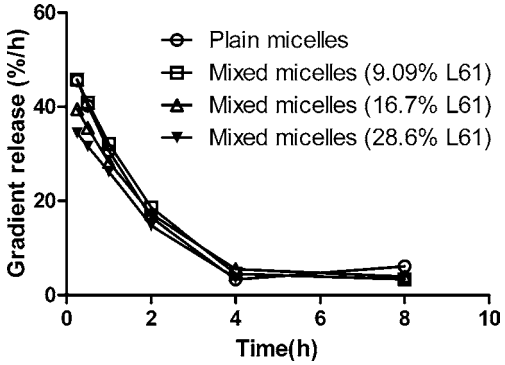

Fig. 4 Gradient release profiles of PTX from plain micelle and mixed micelles in 1 mol/l sodium salicylate solution ($n=4$).

importantly, it was worth noting that PTX-loaded mixed polymeric micelles with lower content of L61 (1.96%, 3.85%) exhibited comparable activity to that observed with Cremophore EL-based PTX formulation in inhibiting the growth of MCF-7/ADR cells. Therefore, it might be suggested that mixed micelles in the present study efficiently deliver PTX to the cells and retain the pharmacological activity of PTX.

R123 Accumulation in MDR Cells

In order to investigate the possible mechanism of enhanced cytotoxicity of PTX for mixed micellar PTX and further demonstrate the effect of MDR reversal to MCF-7/ADR cells of mixed micelles, the effects of plain micelles and mixed micelles on the uptake of R123 by MCF-7/ADR cells were evaluated. The fluorescent dye R123 is commonly used as a fluorescent probe for evaluation of the P-gp-mediated drug efflux in P-gp-expressing MDR cancer cells. This compound preferably accumulates in cells without any substantial loss in its fluorescence intensity. The present work utilized this probe to evaluate the P-gp efflux function in the resistant cells in the presence of mixed micelles. R123 accumulation in MCF-7/ADR cells was examined for five treatment groups:

1. The probe in the assay buffer
2. The probe-loaded plain micelle solution
3. The probe-loaded mixed micelle (1.96% L61) solution
4. The probe-loaded mixed micelle (3.85% L61) solution
5. The probe in 20 μmol/l cyclosporine A

As seen in Fig. 6, significant differences in the effects of polymeric micelles on the MDR cells were observed. First, in the case of R123/plain micelles, R123 uptake was increased by 4.98-fold for MCF-7/ADR cells compared to the assay buffer controls ($P<0.05$). Second, as one might expect, the mixed micelles with 1.96% and 3.85% L61 significantly (6.78- and 11.09-fold) enhanced accumulation of R123 in MDR cells compared to the assay buffer controls ($P<0.01$). Furthermore, the mixed micelles with 1.96% and 3.85% L61 substantially enhanced accumulation of R123 in MDR cells compared to the plain micelles ($P>0.05$ and $P<0.05$, respectively). Excitingly, a remarkable increase in R123 uptake for mixed micelles with 3.85% L61 was observed compared to mixed micelles with 1.96% L61 ($P>0.05$), which was directly correlated to the cytotoxicity results. In conclusion, the increased absorption of the R123 probe in the MDR cells was attributable to the effects of the block copolymer L61 inserted in mixed micelles.

The last treatment group was included in the study as the control. It could also be seen that cyclosporine A, which reverses MDR by inhibiting P-gp in a competitive manner, affected the uptake of P-gp-dependent probe by MDR cells at a lesser degree than mixed micelles with 3.85% L61, although there was a less significant difference ($P>0.05$), suggesting that a small part of R123/mixed micelles got into MDR cells via a P-gp-independent pathway. This was in accordance with the previous report (17). It has been reported that endocytosis, secretion, and some other

Table III IC_{50} and Resistance Reversion Index (RRI) of Various Formulations of PTX Against MCF-7 Cells or MCF-7/ADR Cells by MTT Assay ($n=6$)

Formulation	MCF-7 IC_{50} (ng/ml)	MCF-7/ADR IC_{50} (ng/ml)	RRI
Free PTX	8.74 ± 2.21	11557 ± 452	–
Cremophor-based PTX formulation		1350 ± 126 [a]	8.56
Plain micelles (0% L61)	5.54 ± 1.05 [a]	6122 ± 938 [a,d]	1.89
Mixed micelles (1.96% L61)		1861 ± 310 [a,b,c]	6.21
Mixed micelles (3.85% L61)		1024 ± 233 [a,b,c,e]	11.29

[a] $P<0.001$ *vs.* free PTX.

[b] $P<0.001$ *vs.* plain micelles.

[c] $P>0.05$ *vs.* Cremophor-based PTX formulation.

[d] $P<0.001$ *vs.* Cremophor-based PTX formulation.

[e] $P<0.001$ *vs.* mixed micelles with 1.96% L61.

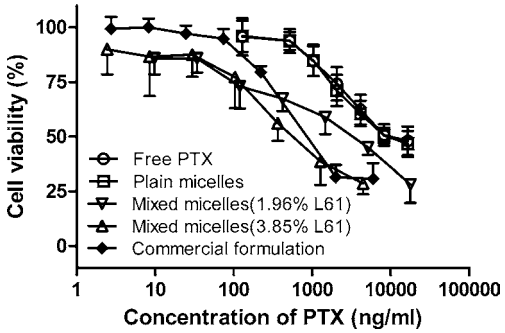

Fig. 5 Cytotoxicity of free PTX, plain micelles and mixed micelles against MCF-7/ADR cells ($n = 6$).

processes related to phase transitions in the plasma membrane are more active in MDR cells than in sensitive ones due to higher membrane fluidity of the former (56). This suggested that the increase in the drug uptake observed in MDR cells in the case of mixed micelles, more accurately, in the presence of L61, might be conditioned by higher flexibility of their membranes. This study reinforced the conclusion that Pluronic block copolymers had pronounced effects, increasing accumulation and permeability of various P-gp-dependent drugs in MDR1-transfected cells, which overexpress P-gp (57,58). Overall, these results provided additional support for the effects of mixed polymeric micelles in the current study for overcoming multidrug resistance in tumor cell lines.

Pharmacokinetics Evaluation

As mentioned above, PTX-loaded mixed polymeric micelles with lower content of L61 (1.96%, 3.85%) exhibited comparable activity to that observed with Cremophore EL-based PTX formulation, and PTX-loaded mixed micelles with 3.85% L61 had significantly better MDR reversal effect to MCF-7/ADR cells as compared with PTX-loaded mixed micelles with 1.96% L61. The mixed micelles with 3.85% L61 were therefore chosen to evaluate pharmacokinetics of PTX in comparison to Cremophore EL-based formulation. The mean plasma concentration-time curves of PTX after intravenous administration of Cremophore EL-based PTX formulation, PTX-loaded plain and mixed micelles to rats at a dose of 5 mg/kg were shown in Fig. 7. The pharmacokinetic parameters of the three formulations calculated according to non-compartmental method were listed in Table IV. No significant difference for AUC, the mean retention time (MRT) and the clearance rate (CL) between any of two formulations were observed ($P > 0.05$), respectively. Overall, mixed polymeric micelle exhibited comparable pharmacokinetic characteristic with Cremophore EL-based formulation. More importantly, it could efficiently deliver PTX to the resistant cells and retain the pharmacological activity of PTX without unfavorable effects resulting from Cremophore EL in Cremophore EL-based PTX formulation.

DISCUSSION

Development of drug resistance to anticancer agents remains one of the major obstacles to cancer chemotherapy (59). One of the main players in MDR mechanisms is a drug efflux transport protein, P-gp, that transports drugs out of tumor cells (6). Increased expression of P-gp in many types of cancers is an adverse prognostic indicator for chemotherapy (60,61). Moreover, high levels of P-gp were found more frequently in recurrent or relapsed cancers as well as induced after initial chemotherapy treatments (62). Therefore, the discovery and use of agents that can prevent the development of drug resistance may result in improved therapeutic outcomes. Some PEO-PPO-PEO triblock copolymers below the CMC, i.e. block unimers, are a novel class of polymeric inhibitors of P-gp that sensitize MDR tumors to doxorubicin, paclitaxel, vinblastine, and other anticancer agents *in vitro* (17,18) and *in vivo* (63,64), and PEO-PPO-PEO triblock copolymeric micelles do not possess such function of P-gp inhibition (16,17). Further, it was reported that PEO-PPO-PEO triblock copolymers with intermediate lengths of PO chains and relatively short EO segments have the highest net efficacy in MDR cells, and L61 was one of the most potent block copolymer sensitizers of MDR (46). Polymeric micelles represent a novel type of nanomedicines that can deliver and release drugs at the target sites (14). In addition, they can also release individual block copolymer molecules which are

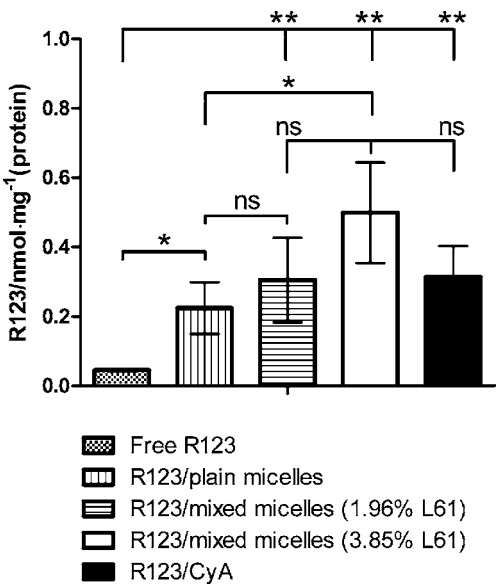

Fig. 6 Intracellular accumulation of R123 in MCF-7/ADR cells ($n = 3$). $^{*}p < 0.05$; $^{**}p < 0.01$; ns, $p > 0.05$.

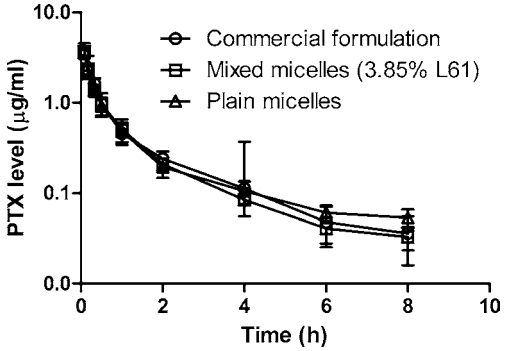

Fig. 7 PTX levels in rat plasma after i.v. administration of the three preparations at the dose of 5 mg/kg. Each point represents the mean ± SD of five rats per time point.

shown to inhibit P-gp and to sensitize MDR cells (14). However, almost all the micelle-forming amphiphilic block copolymers except Pluronic block copolymers could not efficiently sensitize MDR cells (36). Hence, the present work evaluated the ability of MDR reversal of MCF-7/ACR cells *in vitro* by insertion of Pluronic L61 into PTX-loaded mPEG-PLA micelles. L61 was chosen as representative material partially because it readily formed mixed micelles of nanoscaled size with mPEG-PLA.

In order to investigate the effect of the insertion of Pluronic L61 into the mPEG-PLA micelles on MDR reversal of MCF-7/ADR cells, the cytotoxic activities of PTX in micelles were tested by MTT assays against MCF-7 and MCF-7/ADR cells, a human breast cancer cell line and MDR sublines, which have been previously used in studying the anticancer activity and reversal of MDR for PTX (35). In the case of our experiment, the drug resistance of MCF-7/ADR to PTX was first confirmed. Previous reports showed that the drug-resistance indexes of MCF-7/ADR to PTX were 115.5~74.2 (65) and 34.3 (35), respectively, whereas the resistance index of MCF-7/ADR cells in current study was 1322, implying that MCF-7/ADR cells in current study possessed considerably higher drug resistance to PTX compared to the parental lines. This might be partially attributed to the nature of MCF-7/ADR sublines. Although MCF-7/ADR cells were all derived from the parental cells MCF-7 by selection with doxorubicin, different source of MCF-7/ADR cells could have different drug resistance ability. In addition, previous studies (35,49) and our experiments demonstrated that drug-free plain mPEG-PLA micelles, mPEG-PLA/L61 mixed micelles with lower than 3.85% L61 and 1.0% DMSO showed no cytotoxicity against cultured MCF-7 and MCF-7/ADR cells.

As shown in Fig. 5 and Table III, the MDR reversal to MCF-7/ADR cells for plain micellar PTX was observed but only 1.89-fold higher than free PTX. As expected, the resistant sublines (MCF-7/ADR) demonstrated considerably higher response to the cytotoxic action of the mixed micellar PTX compared to that of the plain micellar PTX, indicating that the insertion of L61 could enhance the cytotoxicity of mPEG-PLA micellar PTX to MDR cells. Furthermore, the cytotoxic action of the mixed micellar PTX was related to the L61 content in the tested concentration range. Previous studies reported that unimers of L61 rather than micelles were responsible for the hypersensitization effect of MDR cancer cells, which presumably resulted from the function of P-gp inhibition of L61 (17), and concentrations of L61 ranging from 0.004 mg/ml to 0.4 mg/ml were evaluated to be effective *in vitro* (64). The cellular accumulation of R123 further demonstrated the role of L61 inserted in mixed micelles (Fig. 6). The effect of P85 micelles on cellular accumulation of R123 in BBMEC and Caco-2 cells were reported previously (66–68). These studies demonstrated the different mechanisms of the drug transport with the P85 micelles and unimers due to the fact that the effect of P85 micelles on the uptake of R123 was direction-independent (appeared that it had no relevance to the effects on P-gp efflux system), and, in contrast, P85 unimers increased transcellular flux of R123 in AP to BL direction in BBMEC and Caco-2 cells without affecting the paracellular permeability (68). It was demonstrated that the drug administered to the BBMEC monolayers with the micelles was first absorbed by the cells by vesicular transport mechanism and then recycled out of the cells within the 90 min time interval; in contrast, there was a monotonous increase in R123 accumulation in cells (67,68). Based on these conclusions and the results in current study, we therefore suggested that the L61 unimer-sensitized MDR cells were dissociated from mixed micelles when the micelles disaggregated in cells other than released from mixed micelles as the drug release, resulting in an increase in cytotoxic activity of PTX and overcoming MDR. This could be confirmed by the favorable stability of the mixed micelles in physiological condition in the current study and transport mechanism through cells by endocytosis for polymeric micelles (69).

Table IV Pharmacokinetic Parameters for Different PTX Formulations ($n = 5$)

Preparations	AUC (h·ng/ml)*	CL (ml/h/kg)*	MRT (h) *
Plain micelles	2537.08 ± 364.38	1931.40 ± 318.28	1.20 ± 0.15
Mixed micelles	2351.53 ± 254.55	2065.60 ± 232.49	1.08 ± 0.16
Commercial formulation	2492.50 ± 322.59	1874.97 ± 321.38	1.31 ± 0.10

* $P > 0.05$ between two random preparations

At the same time, it must be noted that IC_{50} of mixed micellar PTX for MCF-7/ADR was about 158-fold greater than that of plain micellar PTX for MCF-7, indicating that the MDR reversal of MCF-7/ADR cells for mixed micelles appeared to be less superior compared with previous report (35). This might be partially attributed to the different drug-resistance ability of MCF-7/ADR sublines as mentioned above. In addition, the L61 content in mixed micelles might be an important influencing factor due to the fact that the content of L61 greatly influenced the MDR reversal effect of mixed micelles. Consequently, the ability of L61 to enhance the drug uptake and to reduce its compartmentalization in MDR cells made this copolymer a potent candidate for a delivery vehicle-forming material for chemotherapeutic agents. The L61 content in mixed micelles will be optimized in further studies.

Previous studies revealed that Cremophor EL-based formulation had some serious associated side effects, including hypersensitivity, neurotoxicity, and nephrotoxicity, and led to extraction of plasticizers from intravenous infusion line and to precipitation on aqueous dilution, which was believed to be caused by the Cremophor EL used for its formulation (37–39). Therefore, it is essential to develop an alternate formulation of PTX free of any drawbacks presented by the presence of Cremophor EL and at the same time overcoming MDR in tumor cells. Polymeric micelles exhibited some advantages. The toxicity of block copolymers to lipid membrane was comparable to that observed with Cremophore EL; however, Cremophore EL exhibited higher cytotoxicity than polymeric micelles (49). In addition, the comparable pharmacokinetic characteristics of the alternative formulation with the Cremophore EL-based commercial formulation are expected. Compared with Cremophore EL-based formulation, there was no significant difference in primary pharmacokinetic parameters, such as AUC, CL and MRT, for plain micelles and mixed micelles, suggesting that PTX-loaded plain micelles and mixed micelles retain the pharmacokinetic characteristics of PTX. However, micelle formulations are often developed with the goal of enhancing drug circulation time by incorporating drugs into so-called stealthy particles which present a hydrophilic shell, such as PEG, on their exterior surface, thus limiting detection by the body's natural clearing mechanisms. Unfortunately, just like our results, pharmacokinetics studies of PTX in diblock copolymer micelles showed that for this type of formulation, long-term circulation did not occur (70). This may be related to the physicochemical property of PTX molecule itself. Just like our observation in the preparation of micelles, the previous report also indicated that the hydrophobic interaction between the drug molecules was greater than that between drug and copolymer due to the high lipophilic character of PTX (35). Therefore, PTX might be faster released from micelles *in vivo*, which was confirmed by the results of micelle stability in plasma. In addition, it could be also due to the fact that PTX in Cremophore EL-based formulation was also solubilized in micelles which are around 13 nm and in similar nanoscaled size.

Overall, mixed micelles could efficiently deliver PTX to the resistant cells and retain the pharmacological activity and pharmacokinetic characteristics of PTX. Thus, mixed micelles in this research would be potential Cremophore EL-free carrier systems for PTX. The studies regarding antitumor efficacy *in vivo* will be carried out to fully characterize the nature of overcoming MDR effect of mixed micelles.

CONCLUSION

In this study, mPEG-PLA micellar PTX and mPEG-PLA/L61 mixed micellar PTX were prepared and characterized. Mixed micellar PTX significantly reduced IC_{50} of PTX in human breast cancer cells MDR sublines (MCF-7/ADR cells) compared to free PTX and plain micellar PTX, and MDR cells were more susceptible to the cytotoxic effects of mixed micellar PTX than the parental cells, suggesting that the insertion of L61 into plain micelles enhanced the cell-killing effect by suppression of the P-gp drug efflux function. Additionally, PTX-loaded mixed polymeric micelles with lower content of L61 exhibited comparable activity to that observed with Cremophore EL-based PTX formulation in inhibiting the growth of MCF-7/ADR cells. Mixed micelles enhanced cellular accumulation of R123 in MCF-7/ADR cells compared to free PTX and plain micellar PTX. Moreover, both plain micelles and mixed micelles retained the pharmacokinetic characteristic of PTX in rats as compared with Cremophore EL-based PTX formulation. Therefore, mPEG-PLA/L61 mixed micelle was a promising alternate nanocarrier for PTX free of Cremphore EL and at the same time overcoming MDR in cancer cells.

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REFERENCES

1. Stein WD, Bates SE, Fojo T. Intractable cancers: the many faces of multidrug resistance and the many targets it presents for therapeutic attack. Curr Drug Targets. 2004;5:333–46.

2. Calderwood SK, Khaleque MA, Sawyer DB, Ciocca DR. Heat shock proteins in cancer: chaperones of tumorigenesis. Trends Biochem Sci. 2006;31:164–72.
3. Kirkin V, Joos S, Zornig M. The role of Bcl-2 family members in tumorigenesis. Biochim Biophys Acta. 2004;1644:229–49.
4. Szakacs G, Paterson JK, Ludwig JA, Booth-Genthe C, Gottesman MM. Targeting multidrug resistance in cancer. Nat Rev Drug Discov. 2006;5:219–34.
5. Duhem C, Ries F, Dicato M. What does Multidrug Resistance (MDR) expression mean in the clinic? Oncologist. 1996;1:151–8.
6. Gottesman MM, Fojo T, Bates SE. Multidrug resistance in cancer: Role of ATP-dependent transporters. Nat Rev Cancer. 2002;2:48–58.
7. Gottesman MM, Pastan I, Ambudkar SV. P-glycoprotein and multi drug resistance. Curr Opin Genet Dev. 1996;6:610–17.
8. Lage H. MDR1/P-glycoprotein (ABCB1) as target for RNA interference-mediated reversal of multidrug resistance. Curr Drug Targets. 2006;7:813–21.
9. Ferry DR, Traunecker H, Kerr DJ. Clinical trials of P-glycoprotein reversal in solid tumours. Eur J Cancer. 1996;32A:1070–81.
10. Krishna R, Mayer LD. Multidrug resistance (MDR) in cancer. Mechanisms, reversal using modulators of MDR and the role of MDR modulators in influencing the pharmacokinetics of anticancer drugs. Eur J Pharm Sci. 2000;11:265–83.
11. Rowinsky EK, Smith L, Wang YM, Chaturvedi P, Villalona M, Campbell E *et al.* Phase I and pharmacokinetic study of paclitaxel in combination with biricodar, a novel agent that reverses multidrug resistance conferred by overexpression of both MDR1 and MRP. J Clin Oncol. 1998;16:2964–76.
12. Thomas H, Coley HM. Overcoming multidrug resistance in cancer: an update on the clinical strategy of inhibiting p-glycoprotein. Cancer Control. 2003;10:159–65.
13. Werle M. Natural and synthetic polymers as inhibitors of drug efflux pumps. Pharm Res. 2008;25:500–11.
14. Kabanov A, Alakhov V. Pluronic block copolymers in drug delivery: from micellar nanocontainers to biological response modifiers. Crit Rev Ther Drug Carr Syst. 2002;19:1–72.
15. Sharma AK, Zhang L, Li S, Kelly DL, Alakhov VY, Batrakova EV *et al.* Prevention of MDR development in leukemia cells by micelle-forming polymeric surfactant. J Control Release. 2008;131:220–7.
16. Kabanov AV, Batrakova EV, Alakhov VY. Pluronic block copolymers for overcoming drug resistance in cancer. Adv Drug Deliv Rev. 2002;54:759–79.
17. Venne A, Li S, Mandeville R, Kabanov A, Alakhov V. Hypersensitizing effect of pluronic L61 on cytotoxic activity, transport, and subcellular distribution of doxorubicin in multiple drug-resistant cells. Cancer Res. 1996;56:3626–9.
18. Alakhov V, Moskaleva E, Batrakova EV, Kabanov AV. Hypersensitization of multidrug resistant human ovarian carcinoma cells by pluronic P85 block copolymer. Bioconjug Chem. 1996;7:209–16.
19. Batrakova EV, Li S, Alakhov VYu, Elmquist WF, Miller DW, Kabanov AV. Sensitization of cells overexpressing multidrug-resistant proteins by Pluronic P85. Pharm Res. 2003;20:1581–90.
20. Batrakova EV, Li S, Elmquist WF, Miller DW, Alakhov VY, Kabanov AV. Mechanism of sensitization of MDR cancer cells by Pluronic block copolymers: Selective energy depletion. Br J Cancer. 2001;85:1987–97.
21. Werle M. Natural and synthetic polymers as inhibitors of drug efflux pumps. Pharm Res. 2008;25:500–11.
22. Kataoka K, Harada A, Nagasaki Y. Block copolymer micelles for drug delivery: Design, characterization and biological significance. Adv Drug Deliv Rev. 2001;47:113–31.
23. Ai H, Flask C, Weinberg B, Shuai X, Pagel MD, Farrell D *et al.* Magnetite-loaded polymeric micelles as ultrasensitive magnetic-resonance probe. Adv Mater. 2005;17:1949–52.
24. Croy SR, Kwon GS. Polymeric micelles for drug delivery. Curr Pharm Design. 2006;12:4669–84.
25. Le Garrec D, Gori S, Luo L, Lessard D, Smith DC, Yessine M-A *et al.* Poly(N-vinylpyrrolidone)-block-poly(D, L-lactide) as a new polymeric solubilizer for hydrophobic anticancer drugs: *in vitro* and *in vivo* evaluation. J Control Release. 2004;99:83–101.
26. Strickley RG. Solubilizing excipients in oral and injectable formulations. Pharm Res. 2004;21:201–30.
27. Kwon GS. Polymeric micelles for delivery of poorly water-soluble compounds. Crit Rev Therap Drug Carrier Syst. 2003;20:357–403.
28. Barratt G. Colloidal drug carriers: achievements and perspectives. Cell Mol Life Sci. 2003;60:21–37.
29. Danson S, Ferry D, Alakhov V, Margison J, Kerr D, Jowle D *et al.* Phase I dose escalation and pharmacokinetic study of pluronic polymer-bound doxorubicin (SP1049C) in patients with advanced cancer. Br J Cancer. 2004;90:2085–91.
30. Matsumura Y, Hamaguchi T, Ura T, Muro K, Yamada Y, Shimada Y *et al.* Phase I clinical trial and pharmacokinetic evaluation of NK911, a micelle-encapsulated doxorubicin. Br J Cancer. 2004;91:1775–81.
31. Mizumura Y, Matsumura Y, Yokoyama M, Okano T, Kawaguchi T, Moriyasu F *et al.* Incorporation of the anticancer agent KRN5500 into polymeric micelles diminishes the pulmonary toxicity. Jpn J Cancer Res. 2002;93:1237–43.
32. Zhang XC, Burt HM, Hoff DV, Dexter D, Mangold G, Degen D *et al.* An investigation of the antitumor activity and biodistribution of polymeric micellar paclitaxel. Cancer Chemother Pharmacol. 1997;40:81–6.
33. Li X, Huang Y, Chen X, Zhou Y, Zhang Y, Li P *et al.* Self-assembly and characterization of Pluronic P105 micelles for liver-targeted delivery of silybin. J Drug Target. 2009;17:739–50.
34. Yang XQ, Deng WJ, Fu LW, Blanco E, Gao JM, Quan DP *et al.* Folate-functionalized polymeric micelles for tumor targeted delivery of a potent multidrug-resistance modulator FG020326. J Biomed Mater Res. 2008;86A:48–60.
35. Wang YZ, Yu L, Han LM, Sha XY, Fang XL. Difunctional Pluronic copolymer micelles for paclitaxel delivery: Synergistic effect of folate-mediated targeting and Pluronic-mediated overcoming multidrug resistance in tumor cell lines. Inter J Pharm. 2007;337:63–73.
36. Chiappetta DA, Sosnik A. Poly(ethylene oxide)–poly(propylene oxide) block copolymer micelles as drug delivery agents: Improved hydrosolubility, stability and bioavailability of drugs. Eur J Pharm Biopharm. 2007;66:303–17.
37. Singla AK, Garg A, Aggarwal D. Paclitaxel and its formulations. Inter J Pharm. 2002;235:179–92.
38. Gelderblom H, Verweij J, Nooter K, Sparreboom A. Cremophor EL: the drawbacks and advantages of vehicle selection for drug formulation. Eur J Cancer. 2001;37:1590–8.
39. Weiss RB, Donehower RC, Wiernik PH, Ohnuma T, Gralla RJ, Trump DL *et al.* Hypersensitivity reactions from taxol. J Clin Oncol. 1990;8:1263–8.
40. Geney R, Ungureanu M, Li D, Ojima I. Overcoming multidrug resistance in taxane chemotherapy. Clin Chem Lab Med. 2002;40:918–25.
41. Zhang X, Jackson JK, Burt HM. Development of amphiphilic diblock copolymers as micellar carriers of taxol. Inter J Pharm. 1996;132:195–206.
42. Kim SC, Kim DW, Shim YH, Bang JS, Oh HS, Kim SW *et al. In vivo* evaluation of polymeric micellar paclitaxel formulation: toxicity and efficacy. J Control Release. 2001;72:191–202.
43. Yang ZL, Li XR, Yang KW, Liu Y. Amphotericin B-loaded poly(ethylene glycol)-poly(lactide) micelles: Preparation, freeze-drying and *in vitro* release. J Biomed Mater Res. 2008;85A:539–46.

44. Wilhelm M, Zhao CL, Wang YC, Xu RL, Winnik MA. Macromolecules. 1999;24:1033–40.
45. Huh KM, Lee SC, Cho YW, Lee J, Jeong JH, Park K. Hydrotropic polymer micelle system for delivery of paclitaxel. J Control Release. 2005;101:59–68.
46. Batrakova E, Lee S, Li S, Venne A, Alakhov V, Kabanov A. Fundamental relationships between the composition of pluronic block copolymers and their hypersensitization effect in MDR cancer cells. Pharm Res. 1999;16:1373–9.
47. Jones MC, Leroux JC. Polymeric micelles—a new generation of colloidal drug carriers. Eur J Pharm Biopharm. 1999;48:101–11.
48. Choa YW, Leeb J, Leeb SC, Huhb KM, Park K. Hydrotropic agents for study of *in vitro* paclitaxel release from polymeric micelles. J Control Release. 2004;97:249–57.
49. Lee SC, Kim C, Kwon IC, Chung H, Jeong SY. Polymeric micelles of poly(2-ethyl-2-oxazoline)-block-poly(ε-caprolactone) copolymer as a carrier for paclitaxel. J Control Release. 2003;89:437–46.
50. Friche E, Jensen PB, Sehested M, Demant EF, Nissen NN. The solvents Cremophor EL and Tween 80 modulate daunorubicin resistance in the multidrug resistant Ehrlich ascites tumor. Cancer Commun. 1990;2:297–303.
51. Hwang M, Ahn C-H, Pine PS, Yin JJ, Hrycyna CA, Licht T *et al.* Effect of combination of suboptimal concentrations of P-glycoprotein blockers on the proliferation of *MDR1* gene expressing cells. Int J Cancer. 1996;65:389–97.
52. Brasitus TA. Modulation of P-glycoprotein-mediated drug transport by alterations in lipid fluidity of rat liver canalicular membrane vesicles. J Biol Chem. 1992;267:24995–5002.
53. Dudeja PK, Anderson KM, Harris JS, Buckingham L, Coon JS. Reversal of multidrug resistance phenotype by surfactants: relationship to membrane lipid fluidity. Arch Biochem Biophys. 1995;319:309–15.
54. Webster L, Linsenmeyer M, Millward M, Morton C, Bishop J, Woodcock D. Measurement of CrEL following Taxol: plasma levels sufficient to reverse drug exclusion mediated by the multidrug-resistant phenotype. J Natl Cancer Inst. 1993;85:1685–90.
55. Webster LK, Linsenmeyer ME, Rischin D, Urch ME, Woodcock DM, Millward MJ. Plasma concentrations of polysorbate 80 measured in patients following administration of docetaxel or etoposide. Cancer Chemother Pharmacol. 1997;39:557–60.
56. Arsenaut AL, Ling V, Kartner N. Altered plasma membrane ultrastructure in multidrug resistant cells. Bichim Biophys Acta. 1988;938:315–21.
57. Batrakova EV, Miller DW, Li S, Alakhov VY, Kabanov AV, Elmquist WF. Pluronic P85 enhances the delivery of digoxin to the brain: *in vitro* and *in vivo* studies. J Pharmacol Exp Ther. 2001;296:551.
58. Evers R, Kool M, Smith AJ, Deemter LV, Haas MD, Borst P. Inhibitory effect of the reversal agents V-104, GF120918 and Pluronic L61 on MDR1 Pgp-, MRP1- and MRP2-mediated transport. Br J Cancer. 2000;83:366–74.
59. Naito S, Koga H, Yokomizo A, Sakamoto N, Kotoh S, Nakashima M *et al.* Molecular analysis of mechanisms regulating drug sensitivity and the development of newchemotherapy strategies for genitourinary carcinomas. World J Surg. 2000;24:1183–6.
60. Fojo AT, Ueda K, Slamon DJ, Poplack DG, Gottesman MM, Pastan I. Expression of a multidrug-resistance gene in human tumors and tissues. Proc Natl Acad Sci USA. 1987;84:265–9.
61. van der Kolk DM, de Vries EG, van Putten WJ, Verdonck LF, Ossenkoppele GJ, Verhoef GE *et al.* P-glycoprotein and multidrug resistance protein activities in relation to treatment outcome in acute myeloid leukemia. Clin Cancer Res. 2000;6:3205–14.
62. Fojo T, Bates S. Strategies for reversing drug resistance. Oncogene. 2003;22:7512–23.
63. Batrakova EV, Dorodnych TY, Klinskii EY, Kliushnenkova EN, Shemchukova OB, Goncharova ON *et al.* Anthracycline antibiotics non-covalently incorporated into the block copolymer micelles: *in vivo* evaluation of anti-cancer activity. Br J Cancer. 1996;74:1545–52.
64. Alakhov V, Klinski E, Li S, Pietrzynski G, Venne A, Batrakova EV *et al.* Block copolymer-based formulation of doxorubicin. From cell screen to clinical trials. Colloids Surf B Biointerfaces. 1999;16:113–34.
65. Kitazaki T, Oka M, Nakamura Y, Tsurutani J, Doi S, Yasunaga M *et al.* Gefitinib, an EGFR tyrosine kinase inhibitor, directly inhibits the function of P-glycoprotein in multidrug resistant cancer cells. Lung Cancer. 2005;49:337–43.
66. Batrakova EV, Han H-Y, Alakhov VYu, Miller DW, Kabanov AV. Effects of Pluronic block copolymers on drug absorption in Caco-2 cell monolayers. Pharm Res. 1998;15:850–5.
67. Miller DW, Batrakova EV, Waltner TO, Alakhov VYu, Kabanov AV. Interaction of Pluronic block copolymers with brain microvessel endothelial cells: evidence for multiple absorption pathways. Bioconjugate Chem. 1997;8:649–57.
68. Batrakova EV, Han H-Y, Miller DW, Kabanov AV. Effects of Pluronic P85 unimers and micelles on drug permeability in polarized BBMEC and Caco-2 cells. Pharm Res. 1998;15:1525–32.
69. Mathot F. A. des Rieux, A. Arien, Y.J. Schneider, M. Brewster, V. Preat. Transport mechanisms of mmePEG750P(CL-co-TMC) polymeric micelles across the intestinal barrier. J Control Release. 2007;124:134–43.
70. Liggins RT, Burt HM. Polyether–polyester diblock copolymers for the preparation of paclitaxel loaded polymeric micelle formulations. Adv Drug Deliv Rev. 2002;54:191–202.