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Difunctional Pluronic copolymer micelles for paclitaxel delivery: Synergistic effect of folate-mediated targeting and Pluronic-mediated overcoming multidrug resistance in tumor cell lines

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Abstract

A significant obstacle for successful chemotherapy with paclitaxel (PTX) is multidrug resistance (MDR) in tumor cells. Micelles and mixed micelles were prepared from Pluronic block copolymer P105 or L101 as PTX delivery systems for overcoming MDR. Both micelle systems were covalently modified with the targeting agent folic acid to recognize and bind a variety of tumor cells via their surface-overexpressed folate receptor. There was an increased level of uptake of folate-conjugated micellar PTX (i.e. FOL-P105/PTX, FOL-PL/PTX) compared to plain micellar PTX (i.e. P105/PTX, PL/PTX) in human breast cancer MDR cell sublines, MCF-7/ADR, and the uptake of folate-conjugated micellar PTX could be inhibited by free folic acid, which suggested that the level of uptake could be mediated by the folate receptor. The cytotoxicity of folate-conjugated micellar PTX in the MDR cell culture model was much higher compared with plain micellar PTX or free PTX, and the plain micellar PTX also has higher cytotoxicity than free PTX. Overall, the MDR cells are more susceptible to the cytotoxic effects of Pluronic micellar PTX than their parental cells. The introduction of folic acid into P105 or PL mixed micelles enhanced the cell-killing effect by active internalization. Increased internalization explained the improved cytotoxicity of the FOL-micellar PTX to tumor cells. We suggest that the combined mechanisms of folate-mediated active internalization and Pluronic-mediated overcoming MDR be beneficial in treatment of MDR solid tumors by targeting delivery of micellar PTX into the tumor cells where folate receptor is frequently overexpressed, reducing accumulation of micellar PTX in other tissues or organs and further reducing side effects and toxicities of the drug.

Keywords: Pluronic block copolymer; Micelles; Multidrug resistance (MDR); Paclitaxel; Tumor cell targeting; Folate

1. Introduction

Paclitaxel (PTX), the first of a new class of microtubule stabilizing agents, has demonstrated significant antitumor activity in clinical trials against a broad range of solid tumors, including refractory ovarian cancer, metastatic breast cancer, non-small-cell lung cancer, AIDS-related Kaposi's sarcoma, head and neck malignancies and other cancers (Rowinsky, 1995; Kim et al., 2001; Singla et al., 2002). PTX is a highly hydrophobic diterpenoid pseudoalkaloid having molecular formula $C_{47}H_{51}NO_{14}$, corresponding to molecular weight of 853 Da (Rowinsky, 1995; Singla et al., 2002). Because PTX has a poor aqueous solubility of approximately 1 μg/ml (Lee et al., 2003a), it is currently formulated as Taxol, a concentrated solution containing 6 mg PTX/ml of Cremophor EL (polyoxyl 35 castor oil) and dehydrated alcohol (1:1, v/v) (Kim et al., 2001). However, intravenous administration of the current Cremophor EL-based formulation in a non-aqueous vehicle may lead to serious side effects in some patients such as hypersensitivity, neurotoxicity, nephrotoxicity, and to extraction of plasticizers from intravenous infusion line and to precipitation on aqueous dilution (Lee et al., 2003a). Due to this the extensive clinical use of this drug is somewhat delayed (Singla et al., 2002).

Another significant obstacle for successful chemotherapy with PTX is multidrug resistance (MDR) in tumor cells. MDR is a phenomenon whereby tumor cells that have been exposed

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to one cytotoxic agent develop cross-resistance to a range of structurally and functionally unrelated compounds (Thomas and Coley, 2003). MDR is often found in many types of human tumors that have relapsed after an initial favorable response to drug therapy. The sensitivity of the MDR tumor cells to antineoplastic agents can decrease significantly, which hinders the efficacy of these drugs in tumor therapy (Alakhov et al., 1996). Two mechanisms of MDR to the taxanes have been characterized. First, some tumors contain alpha- and beta-tubulin with an impaired ability to polymerize into microtubules and have an inherently slow rate of microtubule assembly that is normalized by the taxanes. A second mechanism for the appearance of MDR often results from elevated expression of particular proteins, such as glycoprotein P (P-gp), which can result in an increased efflux of the cytotoxic drugs from the cancer cells, thus lowering their intracellular concentrations. In certain cases, up to 100-fold overexpression of P-gp in MDR cells have been observed (Rowinsky, 1995; Thomas and Coley, 2003).

Due to these problems, there is a need for the development of alternate formulation of PTX having good aqueous solubility, free of any side effects and at the same time overcoming MDR in tumor cells. Thus, a great deal of effort has been directed towards developing more tolerable aqueous-based formulations for PTX that improve the efficacy of PTX in present-day chemotherapy. Several approaches have been employed to deliver PTX by new formulations including parenteral emulsion, mixed micelles, liposomes, microspheres, nanoparticles, complexes with cyclodextrins, pastes, implants, water-soluble prodrugs, and conjugates, polymeric micelles, etc. (Singla et al., 2002; Lee et al., 2003a). Although these current vehicles employed so far have shown a lot of promise to replace the Cremophor EL-based vehicle for PTX delivery, approaches overcoming the MDR of tumor cells to PTX have seldom been considered. Therefore, the challenge now is to develop a new delivery system that consists of aqueous-based vehicles and possesses ability to overcome the MDR of tumor cells for PTX delivery.

Poly(ethylene glycol)-block-poly(propylene glycol)-block-poly(ethylene glycol) (PEO-block-PPO-block-PEO) micelles have been commonly used for solubilization of hydrophobic drugs (Kabanov et al., 2002a,b). Generally, Pluronic block copolymers with concentration above CMC self-assemble into spherical polymeric micelles in water. Hydrophobic drugs may be physically incorporated within the core of the polymeric micelle by hydrophobic interactions. It is found that Pluronics could interact with MDR cancer cells resulting in drastic sensitization of these tumors with respect to doxorubicin and other anticancer agents (Minko et al., 2005). However, formulation for PTX that employed Pluronic copolymers as micelle forming carriers for solubilization of PTX and reversal of MDR tumors have not been reported to the best of our knowledge so far. PTX solubilization capacity and stability of micellar formation is the two important factors that we should consider when we used Pluronic copolymers as micelle forming carriers. In previous work, our group has prepared micellar PTX formulation employing Pluronic P123 and characterized its drug-loading contents and stability (data not shown), which have demonstrated the compatibility between the core of Pluronic micelles and PTX molecules.

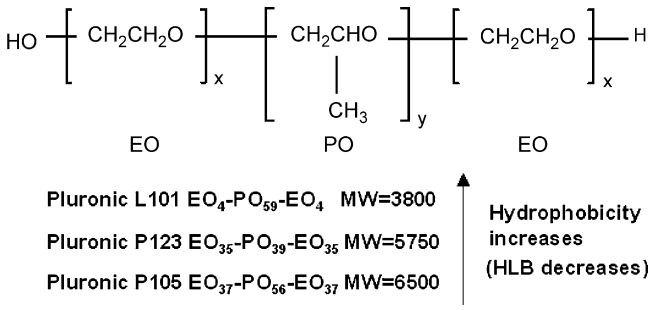

Fig. 1. Chemical structures of Pluronic block copolymers.

In this paper, we have investigated the use of Pluronic P105 and L101 (Fig. 1) as micellar carriers for the solubilization of PTX. We have designed folate-mediated Pluronic micelles in order to improve intracellular internalization of drug-loaded micelles through folate receptor on the tumor cell membrane. And then we compared their cellular uptake and in vitro cytotoxicity assay in MCF-7 and MCF-7/ADR tumor cells between folate-mediated and plain PTX-containing Pluronic micelles. Effect of free folic acid on cellular uptake of micelle formulations in MCF-7/ADR tumor cells and selective uptake of micelle formulations in MCF-7ADR/WI38 co-cultures were also investigated. The objective of this work was to evaluate whether Pluronic micellar PTX possesses ability to overcome MDR, the effect of targeting agent, folic acid, on the cellular accumulation and the cytotoxicity of PTX in MCF-7/ADR. As a result, P105 and P105/L101 micellar PTX have ability to overcome MDR in MCF-7/ADR tumor cells. And folate-mediated Pluronic micelles combined mechanisms of folate-mediated active internalization and Pluronic-mediated overcoming MDR, which further exert synergistic effect on the reversal of MDR in MCF-7/ADR tumor cells.

2. Materials and methods

2.1. Materials

Samples of Pluronic P105 and L101 were kindly supplied by BASF China Ltd. (Shanghai, China). Paclitaxel was purchased from Xi'an Sanjiang Bio-Engineering Co. Ltd. (Xi'an, China). Folic acid, ethylene diamine, *N*-hydroxysuccinimide (NHS), tetrazolium salt MTT, L-glutamine, *N*,*N*′-dicyclohexylcarbodiimide (DCC), fluorescein isothiocyanate (FITC), 4-nitrophenyl chloroformate and folate-free RPMI-1640 medium (R1145) were purchased from Sigma–Aldrich China Inc. (Shanghai, China). Penicillin–streptomycin, fetal bovine serum (FBS) and 0.25% (w/v) trypsin–0.03% (w/v) EDTA solution were provided from Invitrogen Corp. (California, USA). All other reagents were of analytical grade.

2.2. Cell culture

Human breast carcinoma MCF-7 cells (ATCC HTB22) and their MDR MCF-7 ADR cell sublines, derived from the parental cells by selection with doxorubicin, and human lung cell line

WI38 were kindly supplied by Prof. M.L. Wang (Anhui Medical University, Hefei, China). All cells were maintained in folate-free RPMI-1640 (Sigma, R1145) supplemented with 0.3 g/l L-glutamine, 2.0 g/l sodium bicarbonate, 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 U/ml). The only source of folic acid in the media was due to the presence of the 10% fetal bovine serum. The cells were cultured at 37 °C in humidified atmosphere with 5% CO_2. 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 10,000 cells/well on 96-well plates, or 50,000 cells/well on 24-well plates and allowed to grow overnight for reattachment.

2.3. Synthesis of folate-P105 and FITC-P105 conjugates

2.3.1. Activation of P105 with 4-nitrophenyl chloroformate

Pluronic surfactants P105 were activated with 4-nitrophenyl chloroformate as described earlier (Caldwell et al., 1996). Briefly, 1 g of Pluronic P105 was first dissolved in 3 ml benzene and then this solution was slowly added to stirred solution of 4-nitrophenyl chloroformate in 3 ml benzene. The ratio of –OH (P105):–nitrophenyl (4-nitrophenyl chloroformate) for the reaction was kept at 1:3. After 24 h of shaking, the activated P105 was precipitated at least twice using eight-fold excess of petroleum ether (bp 35–60 °C) and was recovered by filtration followed by evaporative removal of the remaining solvent under vacuum overnight. The degree of substitution was determined by measuring the released *para*-nitrophenolate ions in an alkaline solution using a spectrophotometer. An accurately weighed out sample of 4-nitrophenyl chloroformate activated P105 derivative (*p*NP-P105) was dissolved in 0.4% NaOH. After 1 h under rotation, the absorbance was measured at 402 nm using the extinction coefficient of 133.33 ml/(mg cm).

2.3.2. Synthesis of folate-conjugated P105

Aminated folic acids were prepared by the same procedure as reported before (Lee et al., 2003b). Briefly, 450 mg folic acid dissolved in 30 ml DMSO was reacted with DCC (250 mg) and NHS (230 mg) at 50 °C for 6 h. Dicyclohexyl urea (DCU) was removed by filtration and the resulting folate-NHS was mixed with ethylene diamine (600 mg) plus 500 μg pyridine and allowed to react at room temperature overnight. The reaction was confirmed by TLC analysis (silica gel plate, 2-propanol/chloroform, 70:30, v/v). The crude product was precipitated by addition of excess acetonitrile, filtered and washed three times with diethyl ether before drying under vacuum. For further purification, this product was dissolved in 2N HCl and precipitated by adding eight-fold excess of acetonitrile. After filtration, the fine dark yellow powder was dried in vacuum.

Then, folate-conjugated Pluronic P105 (FOL-P105) was synthesized by a modified method as described earlier (Caldwell et al., 1996). Two hundred milligrams of *p*NP-P105 was added to 40 mg of aminated folic acid in 10 ml of 0.1 M NaOH (pH 13). The reaction mixture was stirred and allowed to react further for 4 h at the room temperature. The formed FOL-P105 was finally purified by dialysis against deionized water, followed by lyophilization. The formation of folate-conjugated P105 (FOL-P105) was monitored and confirmed by a gel permeation chromatography (GPC). The conjugation percentage was calculated by determining the amount of folic acid conjugated in FOL-P105. A known amount of dried FOL-P105 was dissolved in dimethylsulfoxide (DMSO) and a UV absorbance value at 365 nm was measured to determine the concentration of conjugated folic acid. Serially diluted concentrations of folic acid in DMSO were used to construct a calibration curve. ^{1}H NMR spectra (400 MHz, DMSO-d_6, ppm) of the modified copolymer showed the peaks at δ (ppm) = 1.02 (d, 3H × 56, –CH_3 of PPO), 2.28 (m, 2H, C_{22}–H_2 of folic acid), 3.33–3.50 (m, 3H × 56, 4H × 74, –CH_2CHO– of PPO and –CH_2CH_2O– of PEO), 4.45 (d, 2H, C_9–H_2 of folic acid), 6.60 (d, 2H, aromatic protons of folic acid), 7.59 (d, 2H, aromatic protons of folic acid), 8.62 (s, 1H, C_7–H of folic acid).

2.3.3. Synthesis of FITC-P105

FITC-labeled P105 were synthesized by a modified procedure as described earlier (Fujita et al., 1999). Briefly, 600 mg of *p*NP-activated P105 were added to 1200 mg of excess ethylene diamine in 15 ml of 0.4% NaOH (pH 13) at room temperature. The reaction mixture was stirred and allowed to react further for 6 h at the room temperature. The released *p*-nitrophenol was registered spectrophotometrically at 402 nm. Unreacted ethylene diamine was removed by evaporation. The formed amino-terminated P105 was finally purified by dialysis against deionized water, followed by lyophilization. Then, the lyophilized amino-terminated P105 was added to 100 mg of FITC in 10 ml DMF and stirred in a nitrogen atmosphere at room temperature in the dark overnight. The mixture was subsequently dialyzed for a week against weakly alkaline aqueous solution (pH 10). The product was freeze-dried to give a yellow viscous liquid and finally purified by GPC. The detection of the fluorescently labeled P105 was performed by absorbance at 405 nm. ^{1}H NMR spectra (400 MHz, DMSO-d_6, ppm) of FITC-labeled P105 showed the peaks at δ = 6.66 (m, 6H, aromatic-H of FITC), 3.33 (m, 3H × 56, –CH_2CHO– of PPO), 3.50 (m, 4H × 74, –CH_2CH_2O– of PEO), 1.02 (d, 3H × 56, –CH_3 of PPO).

2.4. Preparation of paclitaxel-loading block copolymer micelles

Micellar PTX was prepared by thin film method (Zhang et al., 1996). Briefly, 1–10 mg PTX and 225 mg P105 (or P105:L101 = 200 mg:25 mg) samples were dissolved in 10 ml acetonitrile in a round-bottom flask. The solvent was removed by rotary evaporation at 60 °C for about 1 h to obtain a solid PTX/copolymer matrix. Residual acetonitrile remaining in the PTX/copolymer matrix was evaporated overnight under vacuum. Then, the solid PTX/copolymer matrix was preheated in a warm water bath (about 65 °C) to obtain a transparent gel-like sample. The resultant thin film was hydrated with 10 ml H_2O at 65 °C to give a final aqueous concentration of 2.25%

(w/v) of the Pluronic, and the mixture was stirred at 700 rpm for 30 min to obtain a clear micellar solution. Once hydrated, these unincorporated drug aggregates are removed during the filter sterilization process by passing through 0.22 μm filters (Millipore), followed by lyophilization. The content of PTX was determined by RP-HPLC assay after the disruption of the micelles and the solubilization of PTX in acetonitrile.

Folate-conjugated micelles and FITC-labeled micelles were also prepared by the method described above except that Pluronic (P105 or P105/L101) was substituted by adding a mixture of 10 wt% FOL-P105 and 90 wt% Pluronic or a mixture of 0.25 wt% FITC-labeled P105 and 99.75 wt% Pluronic.

In order to improve hydration characteristics of P105 and mixed P105/L101 thin film and rapid reconstitution of the lyophilized Pluronic drug preparations, an appropriate quantity of disaccharides (soluble osmolarity agents, e.g. lactose, sucrose and trehalose, etc.) were added in crystalline form into the acetonitrile during thin film preparation to give a final concentration of 9.5% (w/v). Rotary evaporation was carried out as above, which results in the sugar crystals becoming embedded within the thin film.

2.5. Characterization of micelles

2.5.1. Micelle size determination using laser light scattering

Particle size distribution and mean diameter of the prepared Pluronic micellar PTX were determined by quasi-elastic light scattering using a NICOMP 380 Submicron Particle Sizer (Santa Barbara, CA, USA) equipped with a 5 mW helium–neon laser at 632.8 nm and a temperature controlled cell holder as described previously (Krishnadas et al., 2003). The mean hydrodynamic particle diameter, $\bar{d}_h$ was obtained from the Stokes–Einstein relation using the measured diffusion of particles in solution ($\eta = 0.933$, $T = 23\,^{\circ}C$, $n = 1.33$). Data were analyzed in terms of intensity-weighted NICOMP distributions. Each reported experimental result is the average of at least three $\bar{d}_h$ values obtained from analysis of the autocorrelation function accumulated for at least 20 min.

2.5.2. HPLC assay of solubilized micellar PTX

The amounts of PTX solubilized both in the P105 and P105/L101 was determined by modified RP-HPLC method described previously (Krishnadas et al., 2003). HPLC was carried out using a LC-10AVP HPLC system equipped with a LC-10AT pump and SPD-10A UV detector (Shimadzu, Kyoto, Japan). Twenty microlitres of each sample was injected at least three times into a YMC ODS C18 column (150 mm × 4.2 mm i.d., 5 μm, Japan) preceded by a C18 guard column (Dikma, China). The column temperature was maintained at room temperature. The column was eluted with acetonitrile/35 mM ammonium acetate buffer (50:45, v/v, pH 5) at 1.0 ml/min. The UV absorption at 230 nm was measured. Typical retention time was around 6 min. The drug concentration was calculated from standard curves. The assay was linear over the tested concentration range, and there was no interference of the Pluronic with the assay.

2.5.3. Determination of drug-loading parameters

PTX was extracted from the Pluronic micelles with acetonitrile. The solution was properly diluted prior to HPLC analysis. Drug-loading coefficient (DL) and encapsulation ratio (ER) were calculated as following:

$$\mathrm{DL}\% = \frac{\text{weight of the drug in micelles}}{\text{weight of the feeding polymer and drug}} \times 100\%$$

$$\mathrm{ER}\% = \frac{\text{weight of the drug in micelles}}{\text{weight of the feeding drug}} \times 100\%$$

2.6. Cellular accumulation studies in MDR cells

Accumulation of FOL-micellar PTX (FOL-P105/PTX, FOL-PL/PTX) and plain micellar PTX (P105/PTX, PL/PTX) in receptor-bearing tumor cells was examined using micellar solution containing 50 μg/ml PTX in MCF-7/ADR cell monolayers. Attached MCF-7/ADR cells were treated with 2 ml plain micellar solution or FOL-micellar solution containing 0.65% Pluronic and 50 μg/ml PTX for 1–2.5 h at 37 °C in humidified atmosphere with 5% CO_2. After treatment, cells were washed three times with ice cold PBS buffer, transferred to microcentrifuge tubes and disrupted in a sonicating water bath for 5 min. PTX associated with the cells was measured using RP-HPLC method and obtained values were normalized by protein content in each sample determined using BCA kit according to the manufacturer's protocol. All experiments were repeated in triplicate.

To study cellular uptake of FOL-micellar PTX and plain micellar PTX by the fluorescence microscopy, MCF-7/ADR cell sublines were grown on glass cover slips placed into 6-well tissue culture plates. After the cells reached a confluence of 60–70%, the cells were washed twice with PBS buffer and treated with 2 ml PTX-containing FITC-labeled micelles dispersed in folate-free RPMI 1640 medium at Pluronic concentration of 0.65% and PTX concentration of 50 μg/ml. After 0.5–1.5 h incubation at 37 °C, 5% CO_2, the cover slips were washed six times with PBS buffer and mounted individually cell-side down on fresh glass slides using a fluorescence-free buffered mounting medium. Mounted slides were studied with a Nikon Eclipse E400 microscope under the bright light, or under the epi-fluorescence using FITC filter.

In free folic acid competition studies, uptake of FOL-micellar PTX and plain micellar PTX by receptor-bearing tumor cell was also studied by the method described above except that 1 mM folic acid was added to the incubation medium.

2.7. Cytotoxicity assay in MCF-7 sensitive and MDR cells

Optimum solutions of micellar PTX were used as the test solutions. Serial dilutions of micellar PTX solution were prepared by adding appropriate volume of the micellar solution to 0.26% Pluronic to give a series of PTX concentrations ranging from 0.001 μg/ml to 10 μg/ml and a constant Pluronic concentration (0.26%). Free PTX, dissolved in PBS solution containing 1.0% DMSO as cosolvent, was used as a control. Drug-free

micellar solution and PBS solution containing 1.0% DMSO were also tested as controls.

The cytotoxicity of free PTX, plain micellar PTX and FOL-micellar PTX was assessed by a standard thiazolyl blue tetrazolium bromide (MTT) assay as described previously (Mosmann, 1983). Briefly, MCF-7 and MCF-7/ADR sublines were transferred to flat-bottomed 96-well tissue culture plates (Corning, USA) at a density of 10,000 cells per well 24 h prior to the assay. The culture medium in each well was carefully replaced with 150 μl of medium containing serial dilutions of FOL-micellar PTX, plain micellar PTX or free PTX samples (finally, PTX concentrations ranging from 0.001 μg/ml to 10 μg/ml). After 3 h incubation at 37 °C, the cells were washed three times with sterile PBS and incubated in fresh culture medium for a further 72 h. Then, 20 μl of 5 mg/ml MTT dissolved in PBS was added to each well and the cells were incubated for another 4 h at 37 °C. The medium and indicator dye were then washed out by PBS and the cells were lysed with 150 μl DMSO. The number of viable cells in each well was then determined by absorbance at 570 nm measured on an automated Microplate reader (Bio-Rad, USA) 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 triplicates.

2.8. Selective uptake of micellar formulations in MCF-7ADR/WI38 co-cultures

MCF-7ADR/WI38 co-cultures were prepared by placing simultaneously a mixture of MCF-7 ADR and WI38 in 6-well plates 24 h prior to each experiment. To demonstrate the selective uptake of FOL-micellar PTX, MCF-7ADR/WI38 co-cultures were incubated with FITC-labeled micellar solution containing 50 μg/ml PTX and 0.65% Pluronic. After 1.5 h incubation at 37 °C, the plates were washed four times with 2 ml PBS to remove unbound fluorophores, and then examined on a Nikon Eclipse E400 microscope. The cells were photographed in both the dark field and bright field modes.

2.9. Statistical analyses

All the data are expressed as means ± S.D. Statistical significance was tested by two-tailed Student's *t*-test or one-way ANOVA. P value < 0.05 was considered statistically significant.

3. Results and discussion

3.1. Synthesis of FOL-P105 and FITC-P105

The conjugation of folic acid to Pluronic P105 was performed by activation of the terminal hydroxyl group of P105 by 4-nitrophenyl chloroformate and amination of folic acid by ethylene diamine. The *p*NP-activated P105 was obtained as slight yellow solid with 64% yield. The conjugation of *para*-nitrophenyl-carbonate groups to the PEO ends of P105 was found to be an efficient coupling chemistry and the substitution was essentially independent of the chemical composition of the Pluronic surfactant. The conjugate obtained is quite stable at slightly acidic pH values (around 5); however, its *p*NP group easily interacts with amino groups of low molecular weight and high molecular weight ligands at pH values from 8 and up, with the formation of a stable carbamate (urethane) bond. The relative rates of the coupling and hydrolysis processes can be controlled by the pH value of the reaction system. Even at pH values only slightly above neutral, quite a sufficient number of *p*NP groups is available for ligand binding. Folic acid was aminated with ethylene diamine and then conjugated to the *p*NP-activated polymer through the ethylene diamine spacer. The conjugation of ethylene diamine to folic acid was carried out under proper reaction conditions to modify selectively γ-carboxylic group of folic acid. The α-carboxyl group is, in fact, involved in the interaction with the folate receptor and its modification is detrimental for the biological recognition properties of this vitamin. Since the difficulty of separation of the two folic acid derivatives, the mixture was used for conjugation to the Pluronic P105. The unconjugated folic acid and other chemicals used for the conjugation were thoroughly removed by dialysis against water (MWCO 3000). The conjugation percentage of folic acid to Pluronic P105 was 44.8% on a molar ratio basis.

To introduce FITC at both terminals of the triblock copolymer P105 via a thiocarbonyl linkage, the terminal hydroxyl groups in Pluronic P105 were aminated using the *p*NP-activated Pluronic P105 and ethylene diamine spacer. FITC-P105 was obtained as a slightly yellow powder with the yield of around 34%.

3.2. The preparation and characterization of block copolymer micelles

In general, the 'thin film method' was used to create a large surface area for faster hydration of thin film and form a solution of both the water insoluble drugs and Pluronic. At temperatures above 50 °C, solid Pluronic is in its molten state and will be excellent solvents for water insoluble PTX. When the drug solubilized in solid film of Pluronic is hydrated, micellar solution will be formed and hydrophobic PTX will be spontaneously incorporated into hydrophobic block of Pluronic copolymer. Micelle formation is essentially a reversible aggregation process induced by limited aqueous solubility of the Pluronic. It therefore displays considerable dependency on a number of factors. The nature of the Pluronic and the compatibility between the core of Pluronic micelles and PTX molecules are two important determinants. Those with intermediate water solubility (PEO content), such as Pluronic P123 and P105, gave the most stable formulations on account of having the capacity to form micelles and to provide a hydrophobic environment to maintain the drug in monomeric form. Nagarajan has demonstrated that aromatic hydrocarbons are incorporated in Pluronic micelles to a greater extent than aliphatic hydrocarbons. Pluronic block copolymers have much higher solubilization capacities and are more selective towards aromatic and heterocyclic compounds than towards aliphatic molecules compared to conventional low molecular weight surfactants (Nagarajan and Ganesh, 1996; Nagarajan,

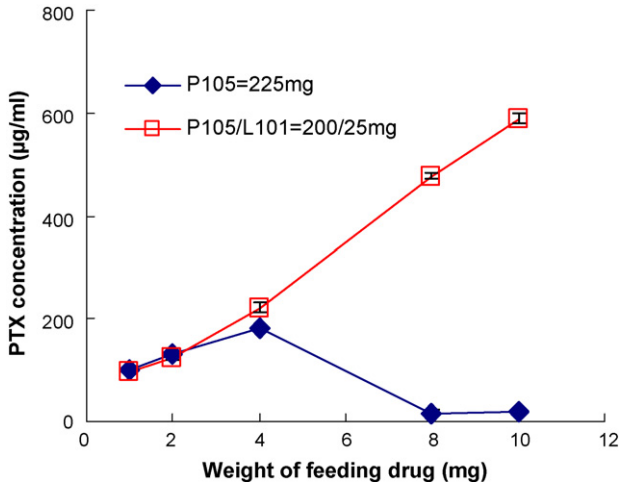

Fig. 2. Effect of weight of feeding drug on solubilization potential of paclitaxel in Pluronic P105 micelles and Pluronic P105/L101 mixed micelles (P105/L101 = 8:1, w/w) at room temperature. Error bars represent standard deviations ($n = 3$ experiments).

1999). Since PTX molecules contain both aromatic and heterocyclic groups, Pluronic micelles appear to be suited for the preparation of pharmaceutical formulations of this drug.

Table 1 shows the results of PTX loading using Pluronic block copolymers P105 and mixed P105/L101. It was found that 2.25% Pluronic P105 solubilized 182.1 ± 4.2 μg/ml PTX when the initial weight ratio of PTX to copolymer was 4 mg:225 mg although PTX has a poor aqueous solubility of approximately 1 μg/ml (Lee et al., 2003a). However, when the ratio exceeded 4 mg:225 mg, it was found that the aggregation of unloaded PTX significantly occurred during the preparation of Pluronic P105 micelles, resulting in the decrease of both the PTX concentration and DL% of PTX in Pluronic P105 micellar solution (Fig. 2, Table 1). It indicated that the hydrophobic interaction between drug molecules was greater than that between drug and Pluronic P105 copolymer due to the high lipophilic character of PTX as the amount of PTX increased.

The solubilization capacity of mixed Pluronic P105/L101 at a mass ratio of 200 mg:25 mg was further evaluated. It was found that 2.25% mixed P105/L101 (P105:L101 = 8:1, w/w) exhibit much greater solubilization capacity (588.1 ± 9.1 μg/ml) compared to P105 micelles. As shown in Fig. 2 and Table 1, PTX concentration and DL% of PTX increased with the ratio of drug to polymer. With feeding drug ranging from 2 mg to 10 mg, PTX concentration in PL/PTX micelle is significantly different from that of corresponding P105/PTX without Pluronic L101 ($P < 0.05$). Especially, with the initial weight ratio of 10 mg:225 mg, the PTX concentration and DL% was 588.1 ± 9.1 μg/ml and 2.5%, respectively, while the highest DL% of PTX in Pluronic P105 micelles just was 0.8% with corresponding PTX concentration of 182.1 ± 4.2 μg/ml.

Pluronic L101 is a hydrophobic Pluronic (HLB = 1) with long PO chains and short EO chains and can usually form lamellar aggregates with larger sizes (ca. 1000 nm) in aqueous solution. Lamellar aggregates formed by hydrophobic Pluronic were found to exhibit a higher solubilization capacity than spherical micelles formed by hydrophilic Pluronic (Oh et al., 2004). Insertion of a small percentage of lamella-forming L101 into a P105 micellar system should increase the volume of the hydrophobic region of the micelle and particle size, which exhibit a higher solubilization capacity for the hydrophobic drug. This is confirmed by the results of this study in which the presence of L101 in the mixed micellar system increased the solubilization potential of PTX by ca. 3.3 times and the micelle size by ca. 7.7 times compared to P105 micellar system. The micelle size of P105 micelles at room temperature was 15–25 nm (Fig. 3A). The mixed micelle size was ca. 185 nm (Fig. 3B), which was intermediate between the sizes of pure Pluronic P105 and L101 micelles. This result that mixed micelle size was ca. 185 nm was basically consistent with the previous results that the size of unloaded P105/L101 (0.1%:0.1%) aggregates was about 171 nm (Oh et al., 2004). It is necessary to note that the aggregation behavior in aqueous solution of binary mixtures of Pluronic is very complicated. Gaisford et al. reported that aggregation behavior of binary Pluronic block copolymers appears to be PPO dependent, Pluronic having similar PPO moieties showing cooperative aggregation and those having different PPO moieties showing non-cooperative binding (Gaisford et al., 1997). The PPO moieties of the selected Pluronic L101 and P105 are 59 and 56, respectively and the length of PPO moieties for both is very close. Hence, the results indicated that binary Pluronic P105/L101 enhanced the solubilization of PTX through forming mixed micelle system in aqueous solution. Furthermore, in a mixed 2% P105/0.25% L101 solution, only one dynamic light scattering peak with a low polydispersity was observed (Fig. 3B), indicating a formation of mixed micelles.

The study of micelle stability was carried out by measuring the size of the particles and concentration of PTX in the dispersions for several days. All samples of both P105/PTX and

Table 1
Drug-loading of paclitaxel in Pluronic P105 micelles and Pluronic P105/L101 mixed micelles (P105/L101 = 8:1, w/w) at room temperature ($n = 3$ experiments)

PTX (mg)	P105 = 225 mg			P105/L101 = 200 mg/25 mg		
	Concentration (μg/ml)	DL%	ER%	Concentration (μg/ml)	DL%	ER%
1	98.9 ± 0.2	0.44	98.9	97.5 ± 1.5	0.43	97.5
2	131.1 ± 1.8	0.58	65.5	124.0 ± 2.5*	0.55	62.0
4	182.2 ± 4.2	0.80	45.5	222.0 ± 9.1*	0.96	55.5
8	16.8 ± 5.3	0.07	2.1	477.1 ± 5.1**	2.04	59.6
10	20.1 ± 0.1	0.08	2.0	588.1 ± 9.1**	2.50	58.8

Note: DL, drug-loading coefficient; ER, encapsulation ratio; PTX, concentration in P105/L101/PTX micelle is significantly different from that of corresponding P105/PTX without Pluronic L101: *$P < 0.05$, **$P < 0.001$.

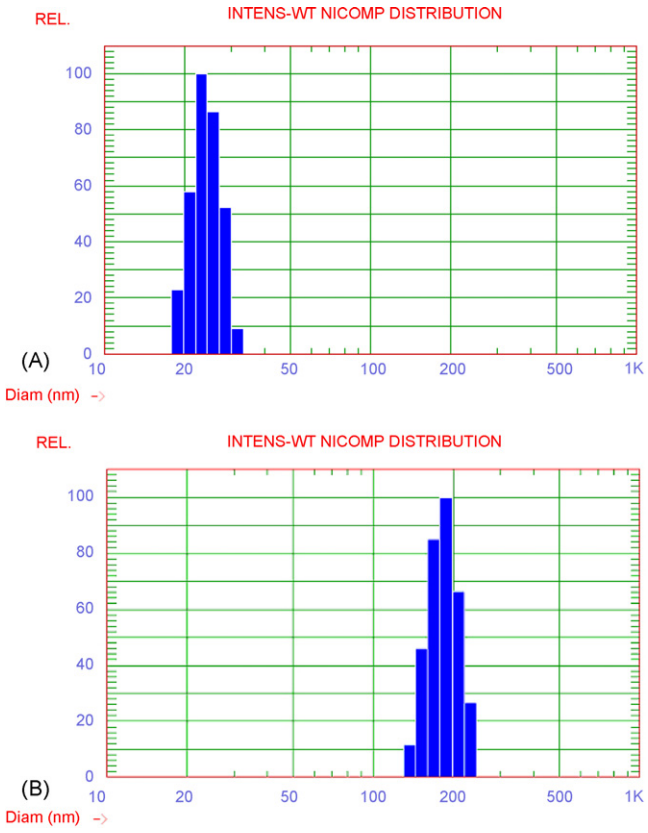

Fig. 3. Representative NICOMP size distribution analysis of Pluronic micellar paclitaxel: (A) P105-paclitaxel, $\bar{d}_h = 23.9 \pm 3.0$ nm; (B) P105/L101 (8:1)-PTX, $\bar{d}_h = 185.3 \pm 21.0$ nm.

PL/PTX phase separated within 2–3 days (data not shown). It was found that higher Pluronic concentrations, higher temperatures and higher ionic strengths would exert better drug stabilization in P105 and mixed P105/L101 micellar PTX. It was also found that incorporation of osmolarity agents into the film, e.g. by having an appropriate quantity of lactose present in crystalline form in the acetonitrile during thin film preparation, helps create a solid that not only facilitates hydration but also allows rapid reconstitution of the lyophilized drug preparations of Pluronic P105 and P105/L101. Substitution of lactose with trehalose resulted in thin films that were not only considerably easier to hydrate but also produced a fluffy lyophilized product that was more readily reconstituted with water.

3.3. Cellular accumulation studies in MDR cells

The cellular uptake of plain micelles and FOL-micelles was evaluated in human breast carcinoma MDR cell sublines, MCF-7/ADR. These cancer cells are expressing a certain level of folate receptors and have been previously studied with folate-mediated polymer-drug formulations (Lee et al., 2003b, 2005)

In order to investigate the effect of time on the FOL-micelles uptake by receptor-bearing cells, MCF-7/ADR cells monolayers were incubated with plain micellar solution and FOL-micellar solution containing 0.65% Pluronic and 50 μg/ml PTX for various time and evaluated for cell-associated PTX by RP-HPLC

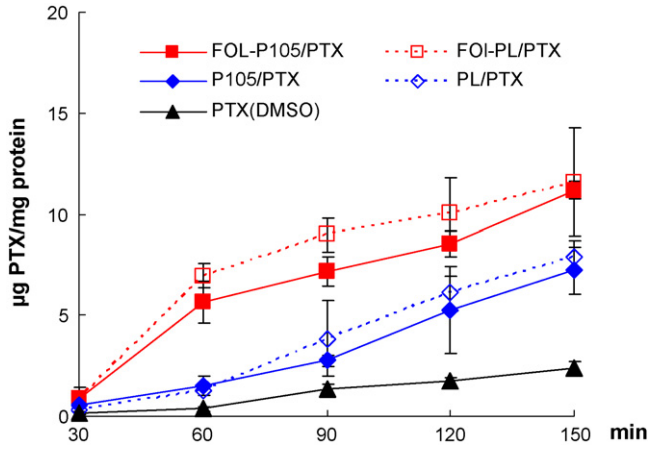

Fig. 4. Uptake of FOL-micellar PTX and plain micellar PTX in MCF-7/ADR cells at different times. MCF-7/ADR cells were incubated with various micellar PTX-containing 0.65% Pluronic and 50 μg/ml paclitaxel at 37 °C for different time. Error bars represent standard deviations ($n = 3$).

method. As shown in Fig. 4, cellular uptake of free PTX was slightly increased during incubation with cell monolayers, which is indicative of the rapid efflux of PTX from MCF-7/ADR cells. However, micellar PTX have showed marked increase of cellular uptake with MCF-7/ADR cells as the time went on. Overall, FOL-micellar PTX had a significant increase in cellular uptake compared to a plain micellar PTX during 2.5 h incubation with MCF-7/ADR cells on account of the effective process of receptor-mediated endocytosis and recycling of folate receptors after internalization. However, uptake rate of FOL-micellar PTX gradually declined after the first 1 h of incubation. This gradual decline in the rate of internalization presumably resulted from saturation of folate receptor-mediated internalization of Pluronic micelles.

In order to evaluate the role of folate receptor in the cellular uptake of FOL-micellar PTX, a competitive binding assay was performed. For these experiments, 1 mM free folic acid was added to the cell culture wells. As shown in Fig. 5, 1 mM free folic acid significantly reduced the PTX uptake in MCF-7/ADR cells incubated with FOL-micellar PTX for 90 min ($P < 0.05$) but had no significant effect in reducing PTX uptake in the case of plain micellar PTX or free PTX. The observations that free folic acid inhibited FOL-micellar PTX uptake suggested that the FOL-micellar PTX might be endocytosed via the folate receptor.

In another experiment, the uptake of the FOL-micellar PTX and plain micellar PTX by MCF-7/ADR cells was visualized using fluorescence microscopy (Fig. 6). MCF-7/ADR cells were incubated with FITC-labeled FOL-micellar PTX or plain micellar PTX for various times (30–90 min). At the same time, 1 mM free folic acid was added to the cell culture wells to observe if free folic acid in medium inhibits uptake of FOL-micellar PTX in MCF-7/ADR cells for the 90 min of incubation. Fig. 6 described uptake of FITC-labeled PL/PTX (Fig. 6A) and FITC-labeled FOL-PL/PTX (Fig. 6B) in MCF-7/ADR cells, as well as effect of free folic acid on uptake of the two micelles. After 30 min of incubation, the absence of cell-associated FITC fluorescence

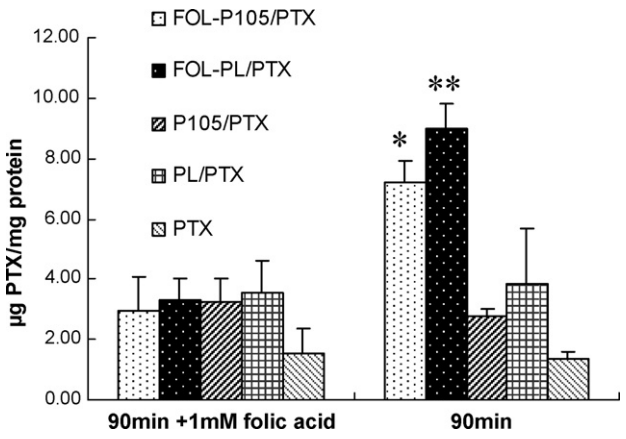

Fig. 5. Inhibition of uptake of FOL-micellar PTX and plain micellar PTX in MCF-7/ADR cells. MCF-7/ADR cells were incubated with various micellar PTX-containing 0.65% Pluronic and 50 μg/ml paclitaxel at 37 °C for 90 min in the presence or absence of 1 mM free folic acid. Error bars represent 1 standard deviation ($n = 3$). $^{*}P < 0.05$: significantly different from the P105/PTX formulation with unpaired Student's *t*-test; $^{**}P < 0.001$: significantly different from the PL/PTX formulation with unpaired Student's *t*-test.

in the control sample (FITC-PL/PTX) in contrast to the slight cell-associated fluorescence in the sample containing FITC-FOL-PL/PTX revealed the dependence of rapid FOL-PL/PTX uptake on the conjugation of folic acid. FOL-PL/PTX had a significant increase in cell-associated FITC fluorescence compared to PL/PTX after 60 min or 90 min of incubation with MCF-7/ADR cells. For free folic acid competition binding, 1 mM free folic acid in cell culture medium reduced the cell-associated fluorescence of FOL-PL/PTX into the level of plain PL/PTX after 90 min of incubation. This observation showed that the FOL-micellar PTX had a significant increase in cell-associated FITC fluorescence compared to the plain PL/PTX, comparable to that observed in cellular PTX accumulation studies in MDR cells using HPLC method.

3.4. Cytotoxicity assay in sensitive and MDR cells

As a next step, we investigated whether the increased cellular PTX accumulation of micellar PTX and FOL-micellar PTX increase their cytotoxicities in tumor cells. To determine the cytotoxic activities of PTX in Pluronic micelles, the formulations were tested against MCF-7 and MCF-7/ADR, 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 (Vredenburg et al., 2001).

MCF-7 or MCF-7/ADR cells grown in 96-well plates were exposed to serial dilutions of micellar PTX or free PTX for 3 h, and cell viability was determined by the MTT assay following 72 h further incubation, as described in Section 2. Serial dilutions of micellar PTX contain 0.26% Pluronic and PTX concentrations ranging from 0.001 μg/ml to 10 μg/ml. PTX concentrations leading to 50% cell-killing (IC_{50}) were determined from concentration-dependent cell viability curves. Empty FOL-Pluronic micellar solution or plain Pluronic micellar solution at the Pluronic concentrations of 0.26% and 1.0% DMSO showed no cytotoxicity against cultured MCF-7 and MCF-7 ADR. These results is roughly consistent with those of Rapoport et al., who reported 0.1% Pluronic solution only caused a mild cytostatic action in A2780 cells (Rapoport et al., 2003).

As shown in Fig. 7, Pluronic micellar PTX had higher cytotoxicities toward cultured MCF-7 ADR cells than that of free PTX solution. However, for FOL-micellar PTX (i.e. FOL-P105/PTX or FOL-PL/PTX), the enhancement in cytotoxicity of PTX in MCF-7 ADR cells was observed compared to plain micellar PTX (i.e. P105/PTX, PL/PTX) and free PTX solution. Effects of the formulations on the resistant cells are expressed in the form of a 'resistance reversion index (RRI)', i.e. ratio of IC_{50} of free PTX solution to that of micellar formulations ($IC_{50\,(\text{free PTX})}/IC_{50\,(\text{micellar PTX})}$) (Kabanov et al., 2002b). In MCF-7 ADR cells, the RRI of FOL-PL/PTX, FOL-P105/PTX,

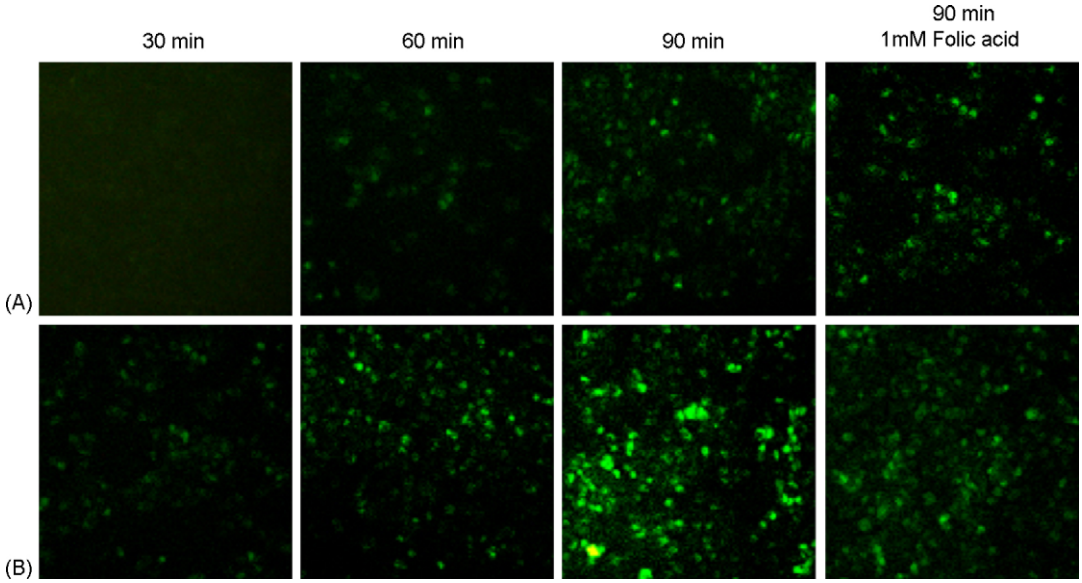

Fig. 6. Uptake of FITC-labeled PL/PTX and FOL-PL/PTX micelles at different times and effect of free folic acid on uptake of the two micelles in MCF-7/ADR cell sublines at 90 min: (A) FITC-labeled PL/PTX micelle; (B) FITC-labeled FOL-PL/PTX micelle.

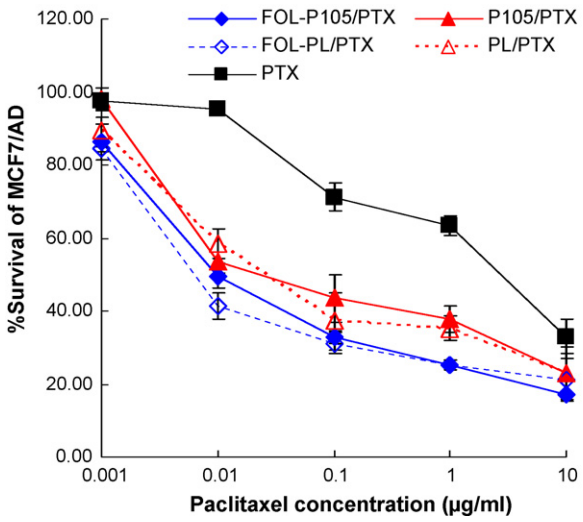

Fig. 7. Cytotoxicity of free PTX, FOL-PL/PTX, FOL-P105/PTX, PL/PTX and P105/PTX against MCF-7/ADR cells ($n = 3$ experiments). Cells were incubated with the drugs for 3 h. Free PTX was dissolved in1.0% DMSO. Pluronic concentration equals 0.26%. Paclitaxel concentration is varied. Error bars represent standard deviations ($n = 3$).

PL/PTX and P105/PTX was ca. 56.2, 45.3, 22.1 and 14.1, respectively (Table 2). By contrast, these Pluronic micellar formulations had a much less significant effect on drug efficacy in the case of sensitive MCF-7 cells. FOL-micellar PTX (i.e. FOL-PL/PTX, FOL-P105/PTX) still had a slightly higher cytotoxicity to cultured MCF-7 cells compared to plain micellar PTX (i.e. PL/PTX, P105/PTX). As shown in Table 2, the IC_{50} of FOL-PL/PTX, FOL-P105/PTX was 5.8 ng/ml and 6.4 ng/ml for MCF-7 cells, respectively, which was about two times lower than PTX in plain micelles (11.4 ng/ml for P105/PTX, 10.8 ng/ml for PL/PTX) and free PTX (12.4 ng/ml). Overall, they had cytotoxic activities toward cultured MCF-7 cells similar to those of free PTX solution.

As a result, the resistant sublines demonstrated considerably higher response to the cytotoxic action of the Pluronic micellar PTX compared to the response in the parental line.

Table 2
IC_{50} (mean ± S.D.) and resistance reversion index (RRI) of various formulations of paclitaxel against MCF-7 Cells or MCF-7/ADR cells

Formulation	MCF7	MCF7/ADR	
	IC_{50} (ng/ml)	IC_{50} (ng/ml)	RRI
Free PTX	12.4 ± 0.7	425.7 ± 69.8	–
P105/PTX	11.4 ± 1.2	30.1 ± 4.4[*]	14.1
PL/PTX	10.8 ± 1.1	19.3 ± 5.4[*]	22.1
FOL-P105	6.4 ± 0.6[*,a,b]	9.4 ± 2.6[*,a,b]	45.3
FOL-PL/PTX	5.8 ± 2.6[**,a,b]	7.5 ± 1.5[*,a,b]	56.2

Note: Resistance reversion index (RRI), i.e. ratio of IC_{50} of free PTX solution and micellar formulations, $IC_{50(\text{free PTX})}/IC_{50(\text{micellar PTX})}$. Multiple comparisons were performed using one-way ANOVA with SNK (Student–Neuman–Keuls) post hoc test ($n = 3$ experiments).

[a] $P < 0.05$: significantly different from the P105/PTX formulation.
[b] $P < 0.05$: significantly different from the PL/PTX formulation.
[*] $P < 0.05$: significantly different from the control values (free PTX).
[**] $P < 0.001$: significantly different from the control values (free PTX).

Compared with plain micellar PTX, higher growth inhibition of FOL-micellar PTX in the sensitive and MDR cells presumably resulted from folate receptor-mediated internalization of Pluronic micelles. The results indicated that PTX-encapsulating FOL-micelles can enhance cytotoxicity of Pluronic micellar PTX in sensitive and ADR cells on account of folate receptor-mediated internalization of micelles and amplification of the drug effect. Overall, the MDR cells are more susceptible to the cytotoxic effects of Pluronic micellar PTX than their parental cells. For FOL-micellar PTX, MDR cells showed higher response to the cytotoxic action than plain micellar PTX, which presumably resulted from synergistic effect of folate-mediated internalization and Pluronic-mediated overcoming MDR in MCF-7/ADR cell lines. These results indicated that FOL-micellar PTX is difunctional micelle system that combines two mechanisms of Pluronic-mediated overcoming MDR and folate-mediated active internalization, and these difunctional micelle systems could be beneficial in treatment of MDR solid tumors.

In fact, cytotoxicity of drug-loading Pluronic micelles depends on the time of cell exposure to micelles and Pluronic concentration. The time dependencies of IC_{50} values for sensitive and MDR cells are different. Alakhov et al. reported the IC_{50} in SKVLB cells (SKOV3 MDR sublines) dramatically decreased approximately 700-fold, while in SKOV3 cells, it changed less significantly during the 150 min incubation with the cells treated with the daunorubicin/Pluronic (Alakhov et al., 1996). In our current work, the duration of cell exposure to Pluronic micellar PTX was 3 h, which was found to be enough to show their ability to significantly reverse drug resistance according to our previous work (data not shown). On the other hand, cytotoxicity of drug-loading Pluronic micelles depends on Pluronic concentration. Kabanov et al. reported cytotoxicity of drug in MDR cells increases with increasing concentrations of Pluronic until CMC is reached and unimer concentration levels off (Kabanov et al., 2002a). Furthermore, Rapoport et al. has demonstrated that the intracellular uptake by the MDR cells slightly increased when drug was delivered in Pluronic micelles in contrast to drug-sensitive cells, and drug encapsulation in Pluronic micelles still noticeably enhanced the inhibition of cell growth in the MDR cells (Rapoport et al., 2003). In our present study, the final Pluronic concentration is 0.26%, which is necessary for hydrophobic PTX to be solubilized and encapsulated in Pluronic copolymers for the preparation process of micellar PTX. Our results of cytotoxicity has shown that Pluronic micellar PTX prepared from a 0.26% Pluronic concentration (above the border of the CMC for Pluronic P105 at 37 °C) still has higher cytotoxic activity in MCF7/ADR cells compared to MCF7 cells.

3.5. Selective uptake of FOL-micellar PTX by MCF-7/ADR cells in MCF-7 ADR/WI38 co-cultures

WI38 cells, a non-transformed human lung cell line, exhibit morphology easily distinguishable from that of MCF-7/ADR cells, human breast carcinoma MCF-7 MDR sublines. WI38 cells are relatively large and have a fibroblastic appearance, while MCF-7/ADR cells grown in the same culture dish are

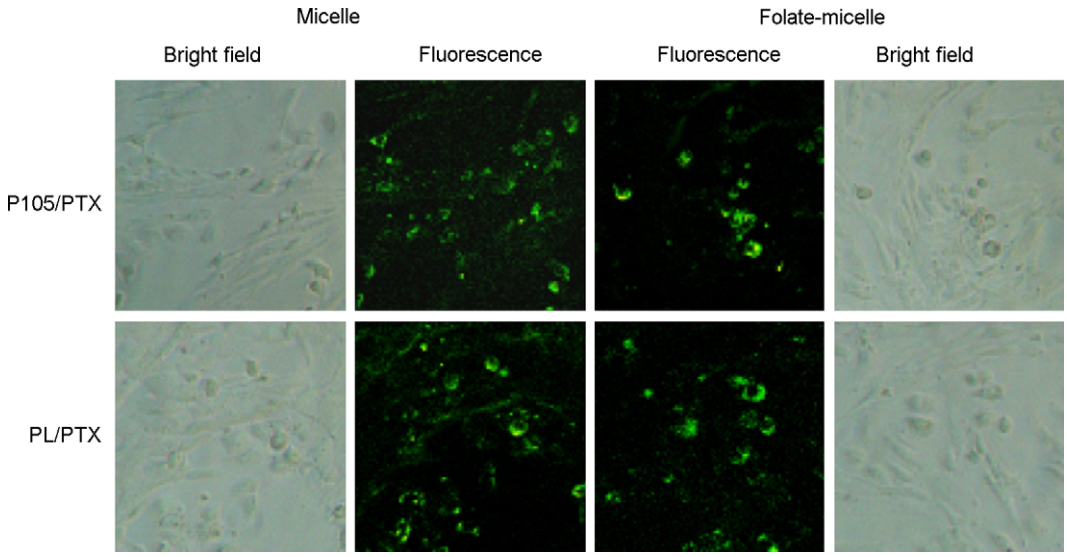

Fig. 8. Selective uptake of FOL-micelles and plain micelles formulation by MCF-7 ADR cells in MCF-7-ADR/WI38 co-cultures at 90 min. MCF-7-ADR/WI38 co-cultures were treated with FITC-labeled FOL-micellar PTX or plain micellar PTX and photographed in both the fluorescence and bright fields mode on a microscope as described in Section 2. (Left) cells treated with FITC-labeled plain micellar PTX; (right) cells treated with FITC-labeled FOL-micellar PTX. (Upper panels) P105 micelles; (lower panels) PL micelles. MCF-7 ADR cells appear as small round or polygonal cells. WI38 are considerably larger with a dendritic or fibroblast-like morphology.

smaller with a round or polygonal morphology (Fig. 8). Furthermore, like most non-transformed cells, WI38 cells lack folate receptors, whereas MCF-7/ADR cells, typical of many cancer cells, greatly express certain level of folate receptors (Lee and Low, 1995; Lee et al., 2005). To determine whether folate receptor might mediate the selective targeting of micelles to MCF-7/ADR cells in the presence of folate receptor-negative cells, MCF-7ADR/WI38 co-cultures were incubated with FITC-labeled FOL-micellar PTX or plain micellar PTX and were examined under a fluorescence microscope.

Fig. 8 is a representative picture of selective uptake of plain micellar PTX and FOL-micellar PTX after 90 min of incubation with MCF-7ADR/WI38 co-cultures. As shown in Fig. 8, although both cells in the co-cultures took up the plain micellar PTX, MCF-7/ADR cells took up more micellar PTX than WI38 cells in the co-cultures treated with both FITC-labeled plain micellar PTXs. And WI38 cells in the co-cultures treated with FITC-labeled plain P105/PTX took up little fluorescence and appeared with a blurry morphology compared to FITC-labeled plain PL/PTX. However, in the case with FITC-labeled FOL-micellar PTX, only MCF-7/ADR cells took up micellar PTX and WI38 cells in the same field did not take up any significant amount of fluorescence in the co-cultures after 90 min. Again, the fluorescence intensities emitted from MCF-7/ADR cells in the co-cultures treated with FOL-micellar PTX and plain micellar PTX were not distinguishable. And both FOL-P105/PTX and FOL-PL/PTX did not show significant difference in targeting uptake when cultured with the co-cultures after 90 min of incubation.

Overall, FITC-labeled plain micellar PTX was taken up by both MCF-7/ADR and WI38 cells, whereas FITC-labeled FOL-micellar PTX was preferentially taken up by the MCF-7/ADR cells after 90 min in the MCF-7ADR/WI38 co-cultures. Although the MCF-7/ADR cells in the co-cultures treated with FITC-labeled FOL-micellar PTX did not show significant increase of fluorescence intensities compared to FITC-labeled plain micellar PTX, the results have still indicated that FOL-micellar PTX may actually be advantageous for targeting delivery of micellar PTX into the tumor cells over-expressing folate receptor and reducing accumulation of micellar PTX in other receptor-negative cells or tissues.

4. Conclusions

In this study, Pluronic P105 and P105/L101 mixed micellar PTX (i.e. P105/PTX, PL/PTX) and the folate-conjugated micellar PTX (i.e. FOL-P105/PTX, FOL-PL/PTX) were prepared and characterized. There was an increased level of uptake of folate-conjugated micellar PTX compared to plain micellar PTX in human breast cancer cells MDR sublines, MCF-7/ADR cells, and the uptake of folate-conjugated micellar PTX could be inhibited by free folic acid, which suggested that the level of uptake could be mediated by the folate receptor. Furthermore, Pluronic micellar PTX significantly reduced IC_{50} of PTX in MDR cells compared to free PTX, and MDR cells are more susceptible to the cytotoxic effects of Pluronic micellar PTX than the parental cells. The introduction of folic acid into P105 or P105/L101 mixed micelles enhanced the cell-killing effect by active internalization. Increased internalization explained the improved cytotoxicity of the FOL-micellar PTX to tumor cells. The combined mechanisms of Pluronic-mediated overcoming MDR and folate-mediated active internalization could be beneficial in treatment of MDR solid tumors by targeting delivery of micellar PTX into the tumor cells where folate receptor is frequently overexpressed, reducing accumulation of micellar PTX in other tissues or organs and further reducing side effects and

toxicities of the drugs. Further studies are in progress to research this difunctional drug delivery system with folate-mediated targeting and Pluronic-mediated overcoming MDR in vivo.

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