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Multifunctional Pluronic P123/F127 mixed polymeric micelles loaded with paclitaxel for the treatment of multidrug resistant tumors

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ABSTRACT

The aim of this study was to exploit the possibility of combination of active targeting function of folic acid by folate receptor-mediated endocytosis and overcoming multidrug resistance (MDR) by Pluronic block copolymers to promote drug delivery to MDR tumor following intravenous administration with paclitaxel (PTX) as model drug. Folic acid functionalized Pluronic P123/F127 mixed micelles encapsulating PTX (FPF-PTX) was firstly developed and tested in vitro and in vivo, while PTX-loaded Pluronic P123/F127 mixed micelles (PF-PTX) and Taxol were used as control. FPF-PTX was about 20 nm in diameter with spherical shape and high encapsulation efficiency. Cellular uptake of FPF-PTX was found to be higher than that of PF-PTX due to the folate receptor-mediated endocytosis effect. In vitro cytotoxicity, cell apoptosis and cell cycle arrest studies also revealed that FPF-PTX was more potent than those of PF-PTX and Taxol. In vivo pharmacokinetic study in rats showed that the polymeric micelles significantly enhanced the bioavailability of PTX (~3 fold) than Taxol. Moreover, in BALB/c mice bearing KBv MDR tumor xenografts, stronger antitumor efficacy was shown in FPF-PTX group, with good correlation between in vitro and in vivo. In conclusion, folate-conjugated Pluronic micelles could be a potential vehicle for delivering hydrophobic chemotherapeutic drugs to MDR tumors.

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1. Introduction

Since liposomes were first described in the 1960s and proposed as carriers of proteins and drugs for disease treatment, nanotechnology has made a significant impact on the development of drug delivery systems. A variety of nanomaterials and devices have been used as delivery vehicles to develop effective therapeutic modalities [1]. One promising nanomedicine-based technology is polymeric micelles, which have been evaluated in several clinical trials as carriers for anti-cancer drugs [2–4]. Polymeric micelles have a core-shell structure which enables the system to incorporate poorly soluble drugs and protect from inactivation in biological media. Due to their small particle size (<100 nm), these systems exhibit many advantages such as targeting ability, long circulation and easy production on effective delivery of drugs [5]. Therefore, polymeric micelles are considered to be an excellent delivery system for hydrophobic anti-cancer drugs.

In this study, we chose Pluronic copolymers as nanocarriers for several reasons. First, Pluronic block copolymers are amphiphilic synthetic polymers containing hydrophilic poly(ethylene oxide) (PEO) blocks and hydrophobic poly(propylene oxide) (PPO) blocks arranged in triblock structure: PEO–PPO–PEO. The hydrophobic PPO segments comprise a hydrophobic core as a microenvironment for the incorporation of lipophilic drugs. The hydrophilic PEO corona prevents aggregation, protein adsorption, and recognition by the reticuloendothelial system (RES) [6]. For instance, a binary mixing system with Pluronic L121/P123 has been developed to produce a stable carrier for hydrophobic agents. A lamellar-forming L121 was incorporated with spherical-forming P123 to increase thermodynamic stability and enhance drug-loading capacity [7]. Additionally, another binary mixed micelles consisting of Pluronic P105 and L101 were also developed for MDR tumor therapy by incorporation with paclitaxel [8]. Second, low cytotoxicity and weak immunogenicity endow Pluronic copolymers in topical and systemic administration. Even though PEO–PPO–PEO copolymers are non-degradable, molecules with the molecular weight less than 15 kDa are usually filtered by the kidney and cleared in urine [6,9–11]. Third, the terminal hydroxyl groups of Pluronic are easy to modify to enhance active tumor targeting ability and improve the physical stability of polymeric micelles [8,12,13]. Recently, to enhance stability of micelles in the blood stream upon dilution and reduce the CMC of the micelles, Pluronic L121 micelles were cross-linked through their hydrophilic shells. To form the crosslinks, the end hydroxyl groups of

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Pluronic L121 were first chemically converted to aldehydes and then bridged via Schiff bases [13]. The Pluronic P123 nano micelles encapsulated with paclitaxel were fabricated by self-assembly means, and then were conjugated with anti-HIF-1a antibody. The targeted micelles could selectively kill cancer cells with over-expression of HIF-1a, and has great potential in clinical tumor therapy [12]. In addition, multifunctional nanoparticle clusters consisting of Pluronic F127 and polyacrylic acid-bound iron oxides conjugated by folic acid were developed by Wang group and it was found to be simultaneously applied as a diagnostic and therapeutic agent that specifically targets cancer cells that overexpress folate receptors in their cell membranes [14]. Fourth, these block copolymers are shown to be inhibitors of P-glycoprotein (P-gp), multidrug resistance proteins (MRPs), and breast cancer resistance protein (BCRP) that sensitize multidrug resistant (MDR) tumors to doxorubicin, paclitaxel, vinblastine and other anti-cancer agents [15]. In particular, doxorubicin loaded mixed micelles composed of Pluronic L61 and F127 (SP1049C) (Supratek Pharma Inc., Montreal, Canada) have already reached Phase III stage [16].

Paclitaxel (PTX) has been one of the most successful anti-cancer drugs, and has shown its potency against a broad spectrum of cancers, especially against lung cancer, metastatic breast cancer and refractory ovarian cancer [17]. However, MDR developed by cancer cells still represents a major challenge in the clinical cure of cancer by PTX alone or in combination with other antineoplastic agents, especially advanced and metastatic forms [18]. In addition, another limitation of the clinical application of PTX is its extremely low solubility in water. To date, there are only two commercially available formulations of PTX are Taxol and Abraxane. Taxol is a concentrated solution composed of a 50: 50 (v/v) mixture of Cremophor EL and dehydrated alcohol, which is diluted 5–20 fold in normal saline or dextrose solution before administration. Unfortunately, serious side effects attributable to Cremophor EL, such as hypersensitivity, nephrotoxicity and neurotoxicity have been reported in Ref. [19]. To overcome the problems caused by Cremophor EL, and to improve the drug efficacy, some research work has been focused on developing new drug delivery systems. Abraxane, an injectable suspension of albumin-bound PTX nanoparticles, is approved for use in patients with metastatic breast cancer or relapse within 6 months of adjuvant chemotherapy [20]. However, Abraxane is so expensive that most of the patients cannot afford it at all. Additionally, there is no evidence reporting that Abraxane has the activity against MDR cancer. Thus, there is great need to develop some novel PTX-loaded nanocarriers to overcome MDR tumors. In this study, we attempted to perform a systematic evaluation of the antitumor efficacy of folic acid functionalized Pluronic P123/F127 mixed micelles encapsulating PTX (FPF-PTX) at the cellular and animal levels by performing intracellular accumulation, sub-cellular distribution, in vitro cytotoxicity, cellular apoptosis and cell cycle assay in KBv cells, FPF-PTX retention in circulation and an in vivo study in xenograft nude mice model.

2. Materials and methods

2.1. Materials

Paclitaxel was purchased from Xi'an Sanjiang Bio-Engineering Co. Ltd. (Xi'an, China). Samples of Pluronic P123, F127 and Cremophor EL were kindly supplied by BASF Ltd. (Shanghai, China). Taxol injection (prepared according to the commercial formulation of Taxol). 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) was purchased from Sigma (St. Louis, MO, USA). Penicillin–streptomycin, RPMI 1640, fetal bovine serum (FBS) and 0.25% (w/v) trypsin–0.03% (w/v) EDTA solution were purchased from Gibco BRL (Gaithersberg, MD, USA). RNase A, propidium iodide (PI), Rhodamine B Isothiocyanate (RITC) and Hoechst 33342 were purchased from Sigma (St Louis, MO, USA). Micro BCA Protein assay kit, Annexin V-FITC Apoptosis Detection kit, ATP assay kit, Mitochondrial membrane potential assay kit with JC-1, MitoTracker Green and TritonX-100 were purchased from Beyotime® Biotechnology Co. Ltd (Nantong, China). Purified deionized water was prepared by the Milli-Q plus system (Millipore Co., Billerica, MA, USA). All other reagents and chemicals were of analytical grade and were used without further purification.

The human lung tumor cell line A-549 and human carcinoma cell line KB were obtained from the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). The multidrug resistant KBv cell line was purchased from Nanjing KeyGen Biotech. Co. Ltd. (Nanjing, China). Culture plates and dishes were purchased from Corning Inc. (NY, USA). The cells were cultured in RPMI 1640 medium, supplemented with 10% FBS, 100 IU/ml penicillin and 100 μg/ml streptomycin sulfate. All the cells were cultured in incubators maintained at 37 °C with 5% CO_2 under fully humidified conditions. All experiments were performed on cells in the logarithmic phase of growth.

Male Sprague–Dawley (SD) rats (230 ± 20 g) and female BALB/c nude mice (20 ± 2 g), supplied by Department of Experimental Animals, Fudan University (Shanghai, China), were acclimated at 25 °C and 55% of humidity under natural light/dark conditions. All animal experiments were carried out in accordance with guidelines evaluated and approved by the ethics committee of the College of Pharmacy, Fudan University (Shanghai, China).

2.2. Synthesis of folate-conjugated Pluronic F127

2.2.1. Synthesis of CDI-activated Pluronic P 127 (F127-CDI)

F127-CDI was synthesized by a modified procedure as described earlier in Ref. [21,22]. Briefly, Pluronic F127 was purified by dissolving in acetone and precipitating into an excess amount of cooled hexane, and dried under vacuum. The purified Pluronic F127 (12.6 g, 1 mmol) was dissolved in dry acetonitrile (15 ml) and added dropwise to an excess amount of *N,N′*- Carbonyldiimidazole (CDI) (1.62 g, 10 mmol) in dry acetonitrile (15 ml) at room temperature during a 2 h period under nitrogen atmosphere. After the addition, the mixture was kept stirring for an additional 4 h. The solution was concentrated in a rotary evaporator, and pour into excess of ethyl ether. This process was repeated three times to remove unreacted CDI. The F127-CDI was dried under a vacuum dehydration and collected as white powder.

2.2.2. Synthesis of amino-terminated Pluronic F127 (F127-NH2)

The F127-CDI (12.7 g, 1 mmol) was dissolved in dry acetonitrile (15 ml) and added dropwise to 10 ml of *1,2*-ethylenediamine at room temperature in 2 h. The mixture was allowed to react overnight. The unreacted ethylenediamine was removed by rotary evaporation and the crude product was poured into an excess amount of ether to obtain the white precipitate. This process was repeated three times, and the F127-NH_2 was dried under a vacuum dehydration and collected as white powder [21,23].

2.2.3. Synthesis of folate-conjugated Pluronic F127 (F127-FA)

The F127-FA was synthesized by a modified method as reported earlier in Ref. [24,25]. The F127-NH_2 (1 g, 0.079 mmol), folic acid (105 mg, 0.237mmol), NHS (60 mg, 0.522 mmol) and DCC (108 mg, 0.522 mmol) were dissolved in 10 ml DMSO in the presence of 0.1 ml Et_3N. The mixture was stirred in a nitrogen atmosphere at room temperature in the dark overnight. Then, the mixture was diluted with 20 ml deionized water and centrifuged in order to separate DCU. The supernatant was further purified by dialysis against deionized water for 2 days, followed by lyophilization.

2.3. Preparation of PTX-loaded mixed micelles

PF-PTX polymeric mixed micelles were prepared by thin-film hydration method as described earlier in Ref. [26]. Briefly, 4 mg of PTX and 270 mg of Pluronic mixture composed of P123 and F127 (2:1, w/w) were dissolved in 10 ml acetonitrile in a round-bottom flask. The solvent was evaporated by rotary evaporation at 50 °C for about 1 h to obtain a solid PTX/copolymer matrix. Residual acetonitrile remaining in the film was removed under vacuum overnight at room temperature. The resultant thin film was hydrated with 4 ml water at 60 °C for 30 min to obtain a micelle solution, which was then filtrated through 0.2 μm filter membrane to remove the uncorporated PTX aggregates, followed by lyophilization. Folate-modified polymeric micelles encapsulating PTX (FPF-PTX) were prepared as described above except that Pluronic F127 was substituted by adding a mixture of 10 wt. % folate-conjugated F127 and 90 wt. % F127 (Fig. 1B) [8,27,28]. In addition, blank RITC-labeled targeted Pluronic P123/F127 mixed polymeric micelles (RITC-FPF) were also prepared as described above except that Pluronic P123 was substituted by a mixture of 10 wt. % RITC-labeled P123 and 90 wt. % P123. RITC-labeled P123 was synthesized as described before in Ref. [29]. The RP-HPLC analysis of PTX in vitro was achieved on a C18 Gemini column (5 μm, 150 × 4.6 mm, Phenomenex, California, USA) with a mobile phase consisting of acetonitrile, and ammonium acetate buffer solution (10 mM, pH 5.0) (50:45, v/v) at a flow rate of 1.0 ml/min. The effluents were monitored at 230 nm and quantified by comparing the peak areas with the standard curve.

2.4. Characterization of PTX-loaded mixed micelles

Drug-loading coefficient (DL%) and encapsulation ratio (ER%) were calculated by the following equations. PF-PTX and FPF-PTX were characterized on a Malvern Zetasizer Naso ZS (Malvern, UK) and by transmission electron microscopy (TEM) on a Hitachi H 600 instrument. In order to create pseudo-sink condition, the in vitro

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