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Effects of paclitaxel nanocrystals surface charge on cell internalization

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ABSTRACT

In this study, we investigated the influence of paclitaxel nanocrystals (PTX-NC) surface charge on cell internalization and cell cytotoxicity. PTX-NCs were prepared using the nano-precipitation method. The surface-modified PTX-NCs were prepared using an absorption method with positively charged poly(allylamine hydrochloride) (PAH) and negatively charged poly(sodium 4-styrenesulfonate) (PSS). The morphologies of the surface-modified and unmodified PTX-NCs were characterized by field emission scanning electron microscopy. An *in vitro* drug release study was performed in phosphate-buffered saline (pH 7.4) containing 0.5% (w/v) Tween 80 for 48 h. Cell internalization was evaluated at time intervals of 0.5, 1, and 2 h, and cell cytotoxicity was analyzed for 24 h using A549 cells. Three different types of PTX-NCs with a mean size of around 300–400 nm were successfully prepared. The zeta-potential revealed PSS-PTX-NCs (-22.7 ± 5.1 mV), PTX-NCs (− 2.4 ± 2.9 mV), and PAH-PTX-NCs (+ 19.3 ± 3.4 mV). The three types of PTX-NC exhibited higher drug release than pure PTX. The positive charge on PTX-NC resulted in higher cell uptake and cell cytotoxicity than the negative charge on PTX-NC. Moreover, the positive charge on PTX-NC showed stronger interactions with bovine serum albumin. In conclusion, the positive charge on PTX-NCs improved cell internalization, cell cytotoxicity, and interactions with bovine serum albumin.

1. Introduction

Paclitaxel (PTX), an anti-cancer drug naturally isolated from the bark of *Taxus brevifolia*, was approved by the Food and Drug Administration for cancer therapy including breast, lung, ovarian, melanoma, and head and neck cancer (US FDA, 1998). However, similarly to many other anti-cancer drugs, the use of PTX is limited because of its inherent properties such as poor solubility (~0.3 μg/mL in aqueous solution) and adverse side effects (Ferrari, 2005; Soga et al., 2005). To solubilize PTX, a mixture Cremophor EL® (polyethoxylated castor oil) and dehydrated ethanol (1:1,v/v) formulated (Taxol®) is used. However, Cremophor EL® causes severe side effects including allergy, hypersensitivity, and pain in many patients (Koudelka and Turánek, 2012; Singla et al., 2002).

Abraxane® approved by FDA in 2005, is similar in efficacy with Taxol® but with reduced side effects and improved solubility of PTX. However, Abraxane® which is an albumin bound formulation is costly. (Stinchcombe, 2007). Therefore, it is necessary to develop a low-cost formulation with improved water solubility of PTX. Numerous studies have attempted to improve the solubility of PTX for drug delivery as a micelle (Zhang et al., 1996), liposome (Dong et al., 2009) polymer nanoparticle (Choi et al., 2014), and drug nanocrystals (Lu et al., 2014).

Drug nanocrystals were developed in the late 1990s, and since then they have been developed at a rapid rate. Currently, commercial products on the market, such as Emend®, Tricor®, and Tridlide®, among others, are prepared using milling technology (Gao et al., 2013). The main advantages of drug nanocrystals include their improved solubility, permeability, and safety. The size reduction of the drug is related to its high solubility and high permeability, as smaller drugs have larger surface areas, improving solubility. In addition, drug nanocrystals have passive targeting effects (Zhang et al., 2015) and do not require additional excipients as Cremophor EL®.

To overcome the poor solubility of PTX, the drug nanocrystal solubilization method has been applied for the delivery of anti-cancer drugs (Hollis et al., 2013; Pereira et al., 2013; Wang et al., 2011). This method has several advantages such as high drug contents and a carrier-free system. There are two main approaches for preparing nanocrystals, i.e., the top-down and bottom-up methods (Chen et al., 2014; Gao et al., 2013; Guo et al., 2015; Li et al., 2015). Briefly, the top-down method uses pearl milling or high-pressure homogenization to break down the drug into smaller-sized particles (Chen et al., 2011; Merisko-Liversidge and Liversidge, 2011; Vidlářová et al., 2016; Yi et al., 2015). The bottom-up method generally employs probe-sonication to inhibit the formation of drug crystals in aqueous solution (Ige et al., 2013; Park et al., 2016; Pattekari et al., 2011).

Polyelectrolytes have negative and positive charges that are used for layer-by-layer deposition onto surfaces, which has been widely studied (Decher, 1997; Sediq et al., 2015). Multilayers are adsorbed by negative

Abbreviations: Paclitaxel nanocrystals, (PTX-NC); field emission scanning electron microscopy, (FE-SEM); distilled water, (DW); bovine serum albumin, (BSA).

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and positive charges as poly(sodium 4-styrenesulfonate) (PSS) and poly(allylamine hydrochloride) (PAH) on particles, respectively (Santos et al., 2015; Zheng et al., 2010). In addition, the layer-by-layer system was shown to increase the solubility of curcumin (Zheng et al., 2010), champtothecin (Shutava et al., 2012), ibuprofen (Santos et al., 2015), and paclitaxel (Agarwal et al., 2008) in nanocrystals. The potential of these multilayer structures for biotechnological and biomedical applications, such as biosensors and carriers for drug delivery, led researchers to extend this technique beyond multilayer structure fabrication based on electrostatic interactions (Antipov and Sukhorukov, 2004; Pastorino et al., 2014).

Binding of a drug to serum albumin is a major problem in pharmaceutical research. The binding of drugs to serum proteins is important, as it affects the activity of drugs and their activity inside the body (Trynda-Lemiesz, 2004; Trynda-Lemiesz and Luczkowski, 2004). It is well-known that drug binding to proteins is an important parameter when determining the pharmacokinetics, restricting the unbound concentration and affecting the distribution and elimination. Macromolecules such as albumin and globulin clearly accumulate in tumor tissues because of enhanced vascular permeability and prolonged retention time in the tumor interstitium following obstruction of lymphatic drainage. Crystallographic studies have demonstrated that binding of paclitaxel to albumin primarily occurs through the core eight-membered taxane ring (He and Carter, 1992; Paal and Shkarupin, 2007).

In this study, a nano-precipitation method (bottom-up) was utilized to reduce the particle size of drug crystals and then the surfaces of drug crystals were modified with PAH and PSS to enhance cell internalization and cell cytotoxicity. Three types of PTX-nanocrystals (NCs) were evaluated with respect to their physical properties, such as morphology and drug release. In addition, three types of PTX-NCs were investigated for cell internalization and cell cytotoxicity in A549 cells, as well as their interaction with BSA.

2. Materials and methods

2.1. Materials

Paciltaxel (PTX)(batch number; F92-11002, USP Paclitaxel) was obtained from the Department of Chemical engineering, Chosun University. PAH (Poly(allylamine hydrochloride))(batch number; MKBV22599V), PSS (Poly(sodium 4-styrenesulfonate)) (batch number; BCBP3081V), Bovine Serum Albumin (BSA) (batch number; 120M1338V), Tetrazolium dye 3-(4, 5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (batch number; MKBW9500V), and DAPI (batch number; 075M4010V) were purchased from Sigma–Aldrich (St. Louis, MO, USA). A549 cells were obtained from College of Pharmacy at Ajou University. Fetal bovine serum (FBS), antibiotics, and Roswell Park Memorial Institute (RPMI 1640) medium were provided by Gibco (Grand Island, NY, USA). Organic solvents were purchased from Samchun Pure Chemicals (Kyunggi-do, Korea).

2.2. Preparation of PTX-NC

PTX-NCs were prepared using a nano-precipitation method (Park et al., 2016). Briefly, PTX (5 mg) was dissolved in ethanol (1 mL). PTX solution was added to distilled water (DW) and subsequently sonicated with a probe-sonicator (VCX 500, Sonics & Materials, Inc., Newtown, CT, USA) at 100 W for 5 min while stirring at 300 rpm. PTX-NCs were collected by centrifugation at 20,000 $\times g$ for 30 min (Avanti® J-E, Beckman Coulter, Brea, CA, USA) and dried under a vacuum in a desiccator for one day.

Surface-modified PTX-NCs were prepared as follows (Choi, 2016). PTX-NCs (3 mg) were re-dispersed in PAH or PSS solution (2 mg/mL) for 1 h while stirring at 300 rpm. Surface-modified PTX-NCs were collected by centrifugation at 12,000 rpm for 30 min (CF-10, Promicrocentrifuge, DAIHAN Scientific Co., Ltd., Gangwon-do, Korea) and then re-dispersed in DW.

For cell internalization, PTX-NCs (with and without surface modification) were re-dispersed in DW (1 mL). Coumarin-6 solution (100 μL) in ethanol (0.1 mg/mL) was added to the PTX-NC suspension while stirring at 300 rpm. Coumarin-6 adsorbed to surface-modified and unmodified PTX-NCs were collected by centrifugation at 12,000 rpm for 30 min and then re-dispersed in DW.

2.3. Morphology observations (FE-SEM)

Morphological evaluation of PTX-NCs and surface-modified PTX-NCs was conducted using field emission scanning electron microscopy (FE-SEM, JSM-6700F, JEOL, Tokyo, Japan). PTX-NC and surface-modified PTX-NC powders were dropped onto a carbon tape. The carbon tape was coated with gold for 2 min under a vacuum. Samples were viewed under an acceleration voltage of 5.0 kV, and the particle sizes of at least 100 particles were measured manually using ImageJ software (National Institute of Health, Bethesda, MD, USA).

2.4. Zeta-sizer and zeta-potential analysis

The particle size of PTX-NCs were measure by Zeta-sizer (Nano-90, Malvern Instruments Ltd., Malvern, UK) and the charge of particle of PTX-NCs were measure by Zeta-potential (Nano-Z, Malvern Instruments Ltd., Malvern, UK). The zeta-sizer and zeta-potential value of each sample were measured in triplicate and the average values were determined.

2.5. In vitro drug release

In vitro drug release studies were performed in pH 7.4 phosphate-buffered saline (PBS) containing Tween 80 (0.5% w/v) for 48 h. The test conditions were 50 rpm shaking speed and 37 ± 0.5 °C. 1 mL of each sample was withdrawn at 0.5, 1, 2, 4, 8, 24, and 48 h. The samples were centrifuged at 12,000 rpm for 30 min and the supernatants were collected. The PTX contents of the sample were determined using high-performance liquid chromatography (HPLC).

2.6. HPLC analysis

The analyses of drug content and drug release of PTX were conducted by HPLC (Waters 2695 Alliance system, Waters, Milford, MA, USA) with an ultraviolet detector (Waters 2484) at a wavelength of 227 nm. A C18 column (Gemini 5u 110A analytical, Intersil OSD-3) operated at 30 ± 0.5 °C was used as the analytical column. The mobile phase was composed of acetonitrile and water (70:30) at a flow rate of 1 mL/min.

2.7. Interaction with BSA

The three types of PTX-NCs were evaluated for their interaction with BSA. PTX-NCs (1 mg) were added to BSA solution (1 mg/mL). The test conditions were 50 rpm of shaking and 37 ± 0.5 °C. All samples were incubated at 0.5, 1, and 2 h and then subjected to centrifugation at 12,000 rpm for 30 min. The supernatants were then collected. BSA contents were quantified using a BCA assay kit (Thermo Fisher Scientific, Waltham, MA, USA) using a multimode microplate reader (Synergy H1 Hybrid Multi-Mode Microplate Reader, BioTek, Winooski, VT, USA) (562 nm).

2.8. Confocal microscopy

Human lung cancer cells (A549) were grown in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum and (5%) antibiotics (100 IU/mL of penicillin G sodium and 100 μg/mL of streptomycin sulfate). For confocal microscopy, A549 cells at an initial density of