International Journal of Pharmaceutics xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical nanotechnology

Free paclitaxel loaded PEGylated-paclitaxel nanoparticles: Preparation and comparison with other paclitaxel systems *in vitro* and *in vivo*

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ARTICLE INFO

Article history:
Received 18 February 2014
Received in revised form 1 May 2014
Accepted 18 May 2014
Available online xxx

Keywords:
Paclitaxel
PEGylated paclitaxel
PEG-PLA
Taxol
Nanoparticles
Antitumor effect

ABSTRACT

Previously, PEGylated paclitaxel (PEG-PTX) was found not favorable as a polymer prodrug because of its poor antitumor efficiency. But surprisingly, it was found in our study that PEG-PTX could form a novel nanoparticle system with free PTX. To address how this system works, we compared PTX loaded PEG-PTX nanoparticles (PEG-PTX/PTX) with PTX loaded PEG-PLA micelles (PEG-PLA/PTX) or PTX injection available (Taxol®) *in vitro* and *in vivo*. Firstly, it was found that PEG-PTX/PTX was more stable in aqueous solution than PEG-PLA/PTX in terms of PTX crystal formation and drug release. Then it was demonstrated that coumarin loaded PEG-PTX nanoparticles had a much higher uptake in MCF-7 cells compared to coumarin loaded PEG-PLA micelles. The *in vivo* imaging study revealed that DIR or DID (near infrared fluorescent substances) loaded PEG-PTX nanoparticles distributed more in tumors in MCF-7 tumor bearing mice than DIR or DID loaded PEG-PLA micelles and solvent system of Taxol®. In the efficacy study with MCF-7 tumor bearing mice, PEG-PTX/PTX showed significantly higher antitumor activity than PEG-PLA/PTX at the same PTX dosage. At the dose of 10 mg free PTX per kg, PEG-PTX/PTX displayed similar efficacy as Taxol® but less toxicity evaluated by the loss of body weight. With the increase of free PTX to 15 mg/kg, PEG-PTX/PTX showed significantly better efficacy than Taxol®. In conclusion, with favorable characteristics in stability, cellular uptake, cytotoxicity, biodistribution, safety and efficacy, PEG-PTX/PTX seems highly potential as a nanocarrier for PTX delivery.

1. Introduction

Paclitaxel (PTX) is an antineoplastic agent that has been demonstrated to have significant activity against a wide variety of tumors, including refractory ovarian cancer, metastatic breast cancer, non-small cell lung cancer and others (Wang et al., 2000). However, PTX is a highly hydrophobic drug (aqueous solubility about 0.3 μg/ml), and the commercially available preparation of Taxol® is a concentrated solution containing 6 mg PTX per ml of Cremophor EL® (polyoxyl 35 castor oil) and dehydrated alcohol (1:1, v/v). Cremophor EL® can lead to hypersensitivity reactions in some cases, which is one of the major obstacles for the success of chemotherapy with PTX (Rowinsky et al., 1993). Therefore, new approaches have focused on developing formulations to solve the hydrophobicity of PTX and the toxic issues associated with Cremophor EL® simultaneously. These novel strategies include preparing water-soluble prodrugs (Mathew et al., 1992; Yang et al., 2012) and nanocarriers such as liposomes (Sharma et al., 1997; Zhou et al., 2013), polymeric micelles (Gaucher et al., 2005; Shin et al., 2009) and nanoparticles (Zhu et al., 2010; Hu et al., 2013).

As nanocarriers, a few PTX formulations have been available in clinic, such as Genexol-PM (Samyang Pharmaceuticals), Abraxane (Celgene Corp.) and Lipisu (Nanjing Pharmaceutical Co., Ltd. Cisco). The sizes of nanocarriers are generally less than 200 nm and they have a propensity to evade scavenging by the mononuclear phagocyte system (Jones and Leroux, 1999). Especially, they are free of Cremophor EL® and comparatively safe. Genexol-PM is a micellular formulation of PTX. It was approved for clinical use in 2007 in Korea (Svenson, 2012). In the US, it has completed a Phase II study and a Phase III trial is ongoing. The polymer used in this formulation is monomethoxy poly(ethylene glycol)-block poly(d, l-lactide) (PEG-PLA), an amphiphilic diblock copolymer. It can

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http://dx.doi.org/10.1016/j.ijpharm.2014.05.032
0378-5173/

solubilize PTX without the hypersensitivity reactions associated with Cremophor EL®. After forming into micelles, it can effectively target therapeutics to their site of action (Kim et al., 2004; Lee et al., 2008). In this study, PTX loaded PEG-PLA micelles (PEG-PLA/PTX) were used as one of the control formulations.

On the other hand, researchers have never stopped modifying the drug itself. Prodrugs are chemical derivatives of an active parental drug with modified properties, such as improved aqueous solubility and biodistribution. Usually, prodrugs have been designed to retain the inherent pharmacological effect of parental drug. Specially, PEGylation is an important strategy in prodrug design (Greenwald et al., 2003). PEGylated PTX (PEG-PTX) is a water-soluble prodrug of PTX (Greenwald et al., 1996), which can be synthesized by combining $mPEG_{5000}$ and PTX using succinic anhydride as the linker (Feng et al., 2002). Unfortunately, this prodrug of PTX has no antitumor activity when administered intravenously alone (Liang et al., 2012), likely due to the failure in effectively release of drug in time.

Although PEG-PTX seems not favorable as a prodrug because of its poor antitumor efficiency, can this amphiphilic polymer be used as a drug carrier? Interestingly, in our study, we found that PEG-PTX do form a novel nanoparticle system with free PTX (PEG-PTX/PTX), which is rather stable. In the attempt to clarify if this system works well, we will compare PEG-PTX/PTX nanoparticles with PTX loaded PEG-PLA micelles (PEG-PLA/PTX) and PTX injection available (Taxol®) in terms of stability, cell uptake, *in vitro* cytotoxicity, *in vivo* tissue distribution, antitumor efficacy and toxicity.

2. Materials and method

2.1. Materials

Paclitaxel was purchased from Shanxi Sciphar Biotechnology Co., Ltd. (Shanxi, China). $mPEG_{5000}$, DID and DIR were obtained from Sigma–Aldrich (St. Louis, MO, USA). $<mPEG_{2000}$-PLA_{2000} (PEG-PLA, Mw/Mn = 1.11) was purchased from Advanced Polymer Materials Inc. (Montreal, Canada). Cremophor EL® was from BASF Corporation of Germany (Local Agent in Shanghai, China). Coumarin was a product from Molecular Probes Inc. (Eugene, Oregon, USA). Succinic anhydride, 1-ethyl-3-(3-dimethyllamino-propyl) carbodiimide hydrochloride (EDC·HCl), and 4-dimethylaminopyridine (DMAP) were obtained from Bo-Mai-Jie Co., Ltd. (Beijing, China). Diisopropylethylamine (DIEA) was purchased from Alfa Aesar (Tianjin, China). Sulforhodamine B (SRB) and Tris base were obtained from Sigma–Aldrich (St. Louis, MO, USA).

The human breast cancer cell line MCF-7 was from the Institute of Basic Medical Science, Chinese Academy of Medical Science (Beijing, China). Cells were cultured in RPMI-1640 (Macgene Biotech Co., Ltd, Beijing, China) supplemented with 10% fetal bovine serum (FBS) and antibiotics (penicillin 100 U/ml and streptomycin 100 mg/ml) at 37° C in 5% CO_2 atmosphere. Female BALB/c nude mice were purchased from Peking University Health Science Center. In this study, all animal experiments were performed in compliance with the institutional ethics committee regulations and guidelines on animal welfare (Animal Care and Use Program Guidelines of Peking University).

2.2. Method

2.2.1. Synthesis of PEGylated paclitaxel (PEG-PTX)

PEG-PTX was synthesized according to Liang et al. reported (Liang et al., 2012). The synthetic route and structure of PEGylated PTX are shown in Fig. 1. First, mPEG-$COCH_2CH_2COOH$ was synthesized (Fig. 1A). In brief, $mPEG_{5000}$ (20 g, 4 mmol) was first stirred in toluene at reflux, then the toluene was evaporated. The mixture of succinic anhydride (2 g, 20 mmol) and pyridine (1 ml) was stirred in $CHCl_3$ (100 ml) at reflux for 48 h. Then, the $CHCl_3$ was evaporated, deionized water was added, and the solution was filtered. The aqueous layer was then extracted with $CHCl_3$ (100 ml) three times. Next, the combined organic layer was washed with brine and dried, filtered, and concentrated to about 30 ml. Ether was added, the solution was filtered, and the residue was dried to yield the product as a white solid. This product was characterized by ^1H NMR and MALDI-TOF-MS.

For the synthesis PEG-PTX (Fig. 1B), mPEG-$COCH_2CH_2COOH$ (560 mg, 0.11 mmol) was stirred in toluene at reflux for 1 h. The toluene was then evaporated, and dropwise EDC·HCl (38 mg, 0.20 mmol) was added to the solution of mPEG-$COCH_2CH_2COOH$ in CH_2Cl_2 (5 ml) followed by cooling at 0° C. After 20 min, PTX (86 mg, 0.10 mmol), DIEA (33 μl, 0.20 mmol) and DMAP (1.2 mg, 0.01 mmol) were added. The mixture was warmed to room temperature and stirred for another 24 h. Next, purification by flash chromatography on silica gel ($CHCl_3$:CH_3OH = 40:1 → 10:1) was performed. Then, the mixture was dissolved in CH_2Cl_2 (20 ml) and washed with brine (10 ml), and the aqueous layer was extracted with CH_2Cl_2 (20 ml). The combined organic layer was dried, filtered and concentrated to yield the product. The product was characterized by MALDI-TOF-MS.

2.2.2. Preparation of PTX loaded PEG-PTX nanoparticles and PEG-PLA micelles

PTX loaded PEG-PTX nanoparticles (PEG-PTX/PTX) was prepared using a thin film hydration method (Wang et al., 2011). First, 10 mg PEG-PTX and 1 mg PTX were dissolved in 3–5 ml of acetonitrile. Second, the organic solvent was removed at room temperature by rotary evaporation for 1 h to form a polymer film. The thin film was warmed to 60° C and hydrated by vortexing with 2 ml of PBS (pH 7.4) at 60° C. Then, the solution was sonicated at 60° C for 5–10 min using a bath type sonicator, untill a transparent clear solution with slightly blue opalescent was obtained. At the same time, blank PEG-PTX nanoparticles were prepared for use as controls. PTX loaded PEG-PLA micelles (PEG-PLA/PTX) were also obtained using the same method, excepting PEG-PTX replaced by PEG-PLA.

2.2.3. Characterization and comparison of blank PEG-PTX nanoparticles and PTX loaded PEG-PTX nanoparticles

The particle sizes of blank PEG-PTX nanoparticles and PTX loaded PEG-PTX nanoparticles were measured by dynamic light scattering (DLS) analysis using a Malvern Zetasizer Nano-ZS (Malvern instruments, UK) at 25° C. The shape and surface morphology of the nanoparticles were investigated by transmission electron microscopy (TEM; JEM-1230, JEOL, Japan) after negative staining with a uranyl acetate solution (1%, w/v). The resultant nanoparticles were further evaluated using an X-ray diffractometer (XRD, Rigaku, Japan) to evaluate the dispersion state of PTX in the nanoparticles. These examinations used Ni-filtered Cu Kα radiation with a 4° C/min scanning rate at room temperature.

2.2.4. Stability comparison of PTX loaded PEG-PTX nanoparticles (PEG-PTX/PTX) and PEG-PLA micelles (PEG-PLA/PTX)

The physical stability of the PEG-PTX/PTX and PEG-PLA/PTX was evaluated according to the appearance of the two systems at different time points under ambient temperature; photographs were also taken with a camera. The microscopic states of the samples were further studied using an optical microscope (Olympus IX71, Japan). During the preparation of PEG-PTX/PTX and PEG-PLA/PTX, scanning electron microscopy (SEM; JSM-5600LV, JEOL, Japan) was applied to observe the thin film state of the co-dissolved PTX and PEG-PTX or PEG-PLA on a pear shaped bottle after organic solvent removal.

Please cite this article in press as: Lu, J., et al., Free paclitaxel loaded PEGylated-paclitaxel nanoparticles: Preparation and comparison with other paclitaxel systems *in vitro* and *in vivo*, Int J Pharmaceut (2014), http://dx.doi.org/10.1016/j.ijpharm.2014.05.032