Development and Evaluation of a Novel TPGS-Mediated Paclitaxel-Loaded PLGA-mPEG Nanoparticle for the Treatment of Ovarian Cancer

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One of the major obstacles to successful paclitaxel (PTX) chemotherapy is toxic side effects, which are often due to the conventional surfactants used, such as Cremophor EL. PTX is characterized by its hydrophobicity and insolubility, which limit its application in ovarian cancer therapy. The aim of this study was to develop Cremophor EL-free PTX-loaded methoxy poly(ethylene glycol)-block-lactic-co-glycolic acid) copolymers (PLGA-mPEG) nanoparticles (NPs) using d-a-tocopheryl polyethylene glycol 1000 succinate (TPGS) as a novel emulsifier. The ability of nanoparticles loaded with paclitaxel (NP-PTX) to inhibit tumor growth was assessed in vitro and in vivo. The acute toxicity of NP-PTX was also evaluated in vivo. We found that paclitaxel was efficiently encapsulated into PLGA-mPEG NPs with a low concentration of TPGS as the emulsifier. The synthesized NP-PTX demonstrated the desired diameter of 80 nm as characterized by transmission electron microscopy. The NP-PTX also exhibited a sustained release of loaded PTX over 4 d with the same chemotherapeutic efficiency and reduced side effects. NP-PTX-treated cells showed slightly lower cytotoxic responses compared with those treated with free PTX at the same concentration. In vivo studies confirmed that NP-PTX significantly enhanced the median lethal dose of paclitaxel by 10-fold, and a similar effect on the inhibition of tumor growth was achieved in nude mice.

Key words nanoparticle; ovarian cancer; paclitaxel therapy

Paclitaxel is characterized by its promising therapeutic index to treat advanced ovarian cancer. However, the therapeutic response is always associated with toxic side effects, such as hypersensitivity reactions, pain at the injection site and dizziness.1-5 In addition, it is difficult to apply paclitaxel to the clinic because of its extremely low solubility in water. Taxol®, a mixture of 50% cremophor EL and 50% dehydrated alcohol (v/v), is the main dosage form for clinical administration of paclitaxel, which requires further 5 to 20-fold dilution with aqueous solutions such as 0.9% sodium chloride. Taxol® is generally given at a dosage of 135 or 175 mg/m2 as a 3 or 24 h infusion every 3 weeks, which is pretreated with corticosteroids, diphenhydramine and H2-receptor antagonists.6 Unfortunately, cremophor EL is not well tolerated which often causes hypersensitivity reactions in some patients.

Therefore, effective paclitaxel chemotherapy depends on the development of novel drug delivery systems. The current approaches mainly focus on developing formulations without cremophor EL and exploring possibilities of large-scale preparation as well as long-term stability.7-9 Different approaches have been developed and evaluated to deliver paclitaxel, such as parenteral emulsions,10-11 mixed micelles,12-14 water-soluble prodrugs15-17 and combined hydroxypropyl-beta-cyclodextrin with poly(anhydride) nanoparticles (NPs).18 Although some approaches have shown advantages in replacing cremophor EL in paclitaxel delivery, dosage forms for clinic treatment are still far away. Substantial effort has been taken to develop more tolerable vehicles, which features as decreased chemotherapy toxicity and improved efficacy of paclitaxel.

Accordingly, there is growing attention in using polymeric NPs as drug delivery vehicles. Methoxy poly(ethylene glycol)-block-(lactic-co-glycolic acid) copolymers (PLGA-mPEG) is one of the popular matrix material for NPs in most cases19-21 due to its biosafety and biodegradability. Poly(ethylene glycol)-block-poly(propylene glycol)-block-poly(ethylene glycol) (PEO-block-PPo-block-PEO) micelles have also been prepared.22 Different surfactants are indispensable in NPs preparation, although diblock copolymers have the potential to form self-assembly micellar structures. They can improve the size, aggregation number and pharmaceutical stability of NPs. Those surfactants include polyvinyl alcohol (PVA) and sodium deoxycholate,19,23 and they need to be removed from NPs suspension by centrifuging and washing. There are two spherical concentric regions in polymeric NPs: one is the densely packed core consisting of hydrophobic blocks and the other is the shell consisting of dense brush of poly(ethylene oxide) (PEO). Hydrophobic drugs may be physically incorporated into the core of polymeric micelles through hydrophobic interactions.24,25

In this study, we develop a cremophor EL-free paclitaxel loading PLGA-mPEG nanoparticle (NP-PTX) by using d-a-tocopheryl polyethylene glycol 1000 succinate (TPGS) as a novel emulsifier. The cytotoxic behavior, acute toxicity and antitumor inhibition of this system were evaluated in vitro and in vivo. TPGS, derived from natural Vitamin E (a-tocopheryl), is a safe and effective form of Vitamin E for reversing or preventing Vitamin E deficiency due to its bioavailability. Varma and Panchagnula reported that TPGS can improve the oral bioavailability by paclitaxel by inhibiting p-glycoprotein-mediated efflux and enhancing permeability.26 Recently TPGS was also reported as an anti-cancer itself by inducing cell cycle arrest and apoptosis selectively in Survivin-overexpressing breast cancer cells.27 It has many potential applications, such as solubilizer, absorption enhancer and vehicle

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for lipid-based drug delivery formulations.28–31 The aim of this study is to achieve optimal homogenetic NPs formation by using TPGS. The common surfactants were substituted by TPGS in preparing PLGA-mPEG NPs, which avoided repetitive removal procedures of centrifugation or dialysis, and surfactant-induced side effects. Nanoparticulate formulation, acting as reservoirs, can protect paclitaxel from epimerization and hydrolysis which facilitates sustained release during treatment. TPGS was a novel efficient emulsifier in inducing NP formation and improving paclitaxel loading efficiency.

In vitro and in vivo evaluation showed that our PLGA-mPEG NP formulation has great advantages in chemotherapy. NPs affected neither cytotoxic activity of paclitaxel in vitro nor inhibition of tumor growth in vivo. These results also demonstrated NPs is characterized by substantially reduced toxicity without causing organ damages. In summary, our newly developed NP-PTXs were useful for a controlled paclitaxel delivery in clinical chemotherapy.

Experimental

Materials Human ovarian adenocarcinoma, IGROV1 cells were kindly provided by Dr. Stephen Collins from UC San Diego (CA, U.S.A.). TPGS was obtained from Jiangsu XiXin Vitamin Co., Ltd. Acetone was purchased from Aladdin Reagent limited corporation (Shanghai, China). PLGA(30)-mPEG(5) (molar ratio of lactide to glycolide, 75/25) was obtained from State Key Lab of Electronic Thin Films and Integrated Devices (University of Electronic Science and Technology of China, Chengdu, China). High-performance liquid chromatography (HPLC) grade acetonitrile (ACN) was purchased from SK Chemicals Co., Ltd. (Korea). The reverse-phase column (250×4.6 mm, 5 µm) was provided from Agilent Technologies Co., Ltd. (U.S.A.). Deionized water was purified by Milli-Q Plus System (Millipore Corporation, U.S.A.).

All animal experiments were reviewed and approved by the Institutional Animal Ethics Committee (IAEC) of the Research Center of Laboratory Animal Science of Zhejiang Chinese Medical University (Hangzhou, China). Permit number is SYXK (ZHE) 2008-0015. ICR mice and BALB/c mice (4–6 weeks old and weighing 18–22 g) were kept at the animal center. NP-PTX was intravenously injected to the animals. Liver, kidney, heart, spleen and lung were collected at designated time point as per the approval of IAEC.

Cell Culture IGROV1 cells were cultured in RPMI 1640 medium (Gibco Inc. Company, U.S.A.) with 10% (v/v) fetal bovine serum (FBS) and antibiotic antimycotic solution (100 ×). Cells were maintained at 37°C in a humidified incubator supplied with 5% CO₂, and dissociated by 0.05% trypsin–ethylenediaminetetraacetic acid (EDTA) (Gibco Inc. Company) for passages.

Preparation of NP-PTX NP-PTX was prepared by the nanoprecipitation method with minor modifications.32 Briefly, 160.1 mg PLGA-mPEG and 40.2 mg paclitaxel were dissolved in 15 mL acetone. The resultant organic solution was added dropwise into 100 mL TPGS solution under rigorous stirring. Nanodroplets were immediately formed due to quick diffusion of acetone to water, and the resultant mixture was introduced into a high pressure homogenizer (Microfluidics M-10L, Microfluidics International Corporation, U.S.A.). Homogenization was performed for five cycles under 12000 psi. Subsequently, acetone was removed by overnight evaporation, which means NP suspension needs no centrifugation or washing as described by Chakravarthi et al.33 The dried NP-PTX can also be obtained by lyophilisation for 48 h. Obtained NPs were directly used in the in vitro and in vivo experiments.

Particle Size and Morphology Observation Each sample was sonicated for 3 min and re-suspended with Milli-Q water in the measuring vessel. The particle hydrodynamic diameter and size distribution were determined by a Coulter Laser Diffraction Particle Size Analyzer (NETASIZER NANO S90, Malvern Instruments Ltd., U.K.). The results were reported as volume size distribution.

The shape and surface morphology of NPs were examined by the transmission electron microscopy (TEM, JEM-1230, JEOL, Japan). Briefly, a drop of NP suspension was placed on copper electron microscopy grids and air-dried before being stained with phosphotungstic acid solution (1% w/v).

Encapsulation Efficiency and Release Study The encapsulation efficiency was defined as the ratio of the actual amount of paclitaxel encapsulated into NPs to the original amount of paclitaxel. Briefly, 5.0 mg of dry NPs was dissolved in 1 mL dichloromethane (DCM), followed by adding 5 mL acetonitrile (ACN) and 5 mL water. The mixture was vigorously shaken for 30 s. A clear solution was achieved by filtering the solution with nitrogen stream until all DCM was removed. Finally, 20 µL of the each sample was analyzed by HPLC twice.

The in vitro release profile of NP-PTX was assessed by determining the residual amount of paclitaxel in NPs. Drug-loaded NPs were put into a centrifuge tube containing 50 mL phosphate buffered saline (PBS, pH 7.4). The vials were incubated at 37°C with reciprocal shaking water bath (120 strokes/min) and monitored for 4 d. NPs samples were collected by ultracentrifugation at designated time points. Supernatant was discarded, and the pellets were washed twice with distilled water and then freeze-dried. Finally, the released profile was measured in triplicate.

Paclitaxel was determined by Beckman System Gold HPLC as described below. A mixture of ACN and water (1:1, v/v) was selected as the mobile phase.34,35 The mobile phase was delivered to a C18 reverse phase column (Agilent Technologies Co., Ltd., U.S.A.) by the pump at 1 mL/min. Paclitaxel was detected using a UV/Vis detector (System Gold 166 Detector) at a wavelength of 227 nm. 32-Karat software was used to measuring the amount of paclitaxel.

Tumor Inhibition Test in Vitro IGROV1 cells were seeded into flat-bottomed 96-well plates at a density of 4×10³ cells/well, and then incubated at 37°C for 24 h in the incubator. Blank NPs, free paclitaxel and NP-PTX ranging from 0.25 to 2 µM were added to the culturing media after cells attached to the plates, respectively. Cytotoxicity was evaluated by 3-(4,5-dimethylthiazol-2-yl)-3,5-diphenyltetrazolium bromide dye (MTT) assay 3 d post the treatment. Briefly, cells were washed with PBS twice after removing the medium and then incubated with MTT solution for 4 h. Next, 200 µL of acidic isopropanol (0.04% HCl) was added to cells after removing the MTT solution. The absorbance was measured at 570 nm using the ELISA microplate reader (TECAN INFINIT M200, Tecan Group Ltd., Switzerland). Percentage of inhibition was calculated as % of inhibition = (1−C1/C0)×100. The C represents absorbance of control, and T represents absorbance of treatment. All measurements were performed three times.
**Toxicity Evaluation** The acute toxicity of NP-PTX was assessed in both male and female ICR mice. Mice were randomly divided into different groups (10 mice of each group). NP-PTX was suspended in 0.8 mL saline and injected to each mouse. Different intravenous injections were performed as below to determine the median lethal dose (LD$_{50}$): 190 mg/kg, 200 mg/kg, 210 mg/kg, 220 mg/kg, 230 mg/kg, 240 mg/kg and 250 mg/kg. The survival of mice was monitored up to 14 d. LD$_{50}$ values were calculated according to the mathematical scheme described by Bliss.

**Tumor Inhibition Test in Vivo** $7 \times 10^6$ IGROV1 cells were subcutaneously injected to the right flank of female nude athymic mice (4–6 weeks, 18–24 g) to establish solid tumors. When the gross tumor reached 100–180 mm$^3$, treatments were started, and this day was designated as day one. All mice were randomly divided into four groups with eight mice per group. A single dose of free paclitaxel or NP-PTX (6 mg/kg) was intravenously administered following the initial treatment. Saline and blank NPs were used as controls. The same dosage was given for 5 d (one dose/per day).

Tumor size and body weight of mice were individually measured every 4 d until the animals were sacrificed. Tumor size was measured by calipers, and tumor volumes were calculated using the formula: $V=\frac{a b^2}{2}$, where $a$ represents the longest diameter, and $b$ represents the diameter orthogonal to $a$ (mm).

**Hematoxylin and Eosin (H&E) Staining** Mice were sacrificed by inhalational anesthetics on day 25. Heart, lung, liver, spleen and kidney were collected for histological examination. Tissues were fixed in 10% neutral-buffered formalin and embedded in paraffin. Five micrometer sections were stained with H&E, and images were taken at 400× magnification. Detailed histological and morphological observations were evaluated by two independent pathologists at Zhejiang Chinese Medical University.

**Cell Death Detection in Vivo** Cell death was assessed by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay. Briefly, tissue sections were dewaxed and rehydrated according to the standard protocol. Slides were washed twice with PBS and then incubated with Proteinase K (Beyotime Institute of Biotechnology, China) working solution for 30 min at room temperature. After rinsing, slides were incubated with reaction mixture for 1 h at 37°C in the dark. Slides were mounted after rinsing and then analyzed under an OLYMPUS IX-71 microscope (U.S.A.) with excitation and emission wavelengths of 488 nm and 520 nm (green), respectively.

**Statistical Analysis** Results were expressed as mean±S.D. unless indicated otherwise. All statistical results were analyzed by SPSS program 11.0 (IBM, U.S.A.). Variance analysis was used to assess the difference in tumor volume and body weight between the treatment and control groups. A $p$ value less than 0.05 was considered as statistically significant.

**Results** TPGS Improved the Formation of NP-PTX Complex
Firstly, PLGA-mPEG based diblock copolymers were dissolved in acetone and then added dropwise into low concentration TPGS aqueous solution (0.035%, w/v). PLGA-mPEG NPs was obtained by microfluidic homogenizer. As shown in Fig. 1, particles were monodisperse in distilled water. The size of NPs was characterized as 128 nm for blank NPs (160.1 mg PLGA-mPEG and 0.035% TPGS), and 168 nm for NP-PTXs (160.1 mg PLGA-mPEG, 40.2 mg PTX and 0.035% TPGS), respectively (Table 1). The results indicated that the particle size of NP-PTX was slightly larger than that of blank NPs. This is due to the fact that incorporating hydrophobic
We first investigated the cytotoxicity of blank PLGA-mPEG NPs in IGROV1 cells, and as shown in Fig. 3A, the finding exhibited that up to a dosage of 80 µg/mL, blank PLGA-mPEG NP showed no obvious inhibition in IGROV1 cell proliferation for 72 h, indicating its safety as drug delivery vehicles. Furthermore, in Fig. 3B, the cytotoxicity of NP-PTX was evaluated in IGROV1 cells at multiple dosages, in comparison with free paclitaxel. As a result, NP-PTX showed equivalent inhibition efficacy to free paclitaxel in IGROV1 cell proliferation. Our data indicated that paclitaxel can be continuously released from NP-PTXs, whereas does not decrease its cytotoxicity against IGROV1 cells.

**Safety Profile of NP-PTX** Toxic side effects induced by conventional surfactants are the major obstacles for successful paclitaxel chemotherapy. Progress has been made to reduce the toxic side effects.20–22,24,25,28–30 Acute toxicity experiments were performed to evaluate drug safety by measuring the LD50. ICR mice were intravenously injected with either NP-PTX or free paclitaxel on a single dose schedule. The LD50 of NP-PTX treated groups (246.85 mg/kg) was over 10-fold higher than that of paclitaxel treated groups (23.58 mg/kg) as shown in Table 3. We further investigated the organ toxicity of NP-PTX by evaluating morphology of main organs, including heart, liver, lung, kidney and spleen after the consecutive five administrations. As a result, no obvious histopathological damage was observed in any of NP-PTX treated groups compared with that of control groups. Further more, alanine amino transferase (ALT), aspartate transaminase (AST) serum values remained within the normal range at 24 h after receiving the 6-mg/kg dose (Table 3). In comparison, morphology of liver in free paclitaxel treated groups was significantly altered with necrosis (Fig. 4), indicating a severe organ toxicity. Our data demonstrated that there was a significant decreased toxicity of paclitaxel encapsulated in NPs, which composed of di-block copolymers with an average molecular weight of 37000 and TPGS.

There are two major reasons that explain the reduced toxicity of NP-PTX: one is the usage of non-toxic TPGS to replace toxic Cremophor EL and the other is controlled release of paclitaxel from biodegradable NPs. There is no need to pre-treat animals with corticosteroids and anti-histamine drugs. In summary, NP-PTX was significantly tolerated *in vivo* by controlled release of paclitaxel.

**In Vivo Efficacy Studies** One single dose of paclitaxel or NP-PTX was injected intravenously to athymic mice at days 1, 6, 12, 18 and 24, respectively. Tumor volume and weight loss of mice were used to measure the antitumor effect of NP-PTX (Fig. 5A). Tumor volume was steadily increased in two control groups (saline and blank NPs) throughout a 24-d post-treatment period, and no significant difference was found between these two groups \(p>0.05\). It clearly indicated that blank NPs had no inhibition on tumor growth. Interestingly, free paclitaxel significantly delayed tumor growth compared with those in control mice \(p<0.05\). NP-PTX of same dosage can also of a 47-year-old woman.40 We investigated the cytotoxicity of blank PLGA-mPEG NPs in IGROV1 cells, and as shown in Fig. 3A, the finding exhibited that up to a dosage of 80 µg/mL, blank PLGA-mPEG NP showed no obvious inhibition in IGROV1 cell proliferation for 72 h, indicating its safety as drug delivery vehicles. Furthermore, in Fig. 3B, the cytotoxicity of NP-PTX was evaluated in IGROV1 cells at multiple dosages, in comparison with free paclitaxel. As a result, NP-PTX showed equivalent inhibition efficacy to free paclitaxel in IGROV1 cell proliferation. Our data indicated that paclitaxel can be continuously released from NP-PTXs, whereas does not decrease its cytotoxicity against IGROV1 cells.

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significantly reduce the tumor growth compared with control group administered with blank NPs. However, its antitumor effect was similar to free paclitaxel treated group ($p > 0.05$).

Figure 5B showed that animal weights were decreased in all groups over time, and no significant difference was observed between groups ($p > 0.05$). In addition, the skin around injec-

Table 3. Toxicity of Paclitaxel and NP-PTX

<table>
<thead>
<tr>
<th>Tested nanoparticles</th>
<th>LD$_{50}$ (mg/kg)</th>
<th>ALT (u/L)</th>
<th>AST (u/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
<td>Male + Female</td>
</tr>
<tr>
<td>NP-PTX</td>
<td>230.42</td>
<td>246.85</td>
<td>238.07</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>22.27</td>
<td>23.58</td>
<td>22.83</td>
</tr>
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Each group included 10 mice; LD$_{50}$ represented as median lethal dose. Data were collected 24h after free paclitaxel (6mg/kg) and NP-PTX (6mg/kg on the basis of paclitaxel) were given to mice ($n=10$).
tion sites was intact without edema or ulceration. Microscopic evaluation of apoptosis by TUNEL staining was observed in athymic mice bearing IGROV1 xenografts when treated with saline, blank NPs, free paclitaxel and NP-PTX at five doses (Fig. 6). From the average apoptotic cell counts calculated on the basis of TUNEL staining, NP-PTX demonstrates the same antitumor activity to free paclitaxel with decreased organ toxicity (Fig. 6B).

Discussion

Our proof-of-concept evaluation in vitro and in vivo showed that a novel method to prepare NP-PTX which combined the merits of TPGS and biodegradable NPs has been successfully developed in our laboratory. NP-PTX was characterized by smooth surface and relatively uniform size. NP-PTX demonstrated tremendous advantages versus the conventional free paclitaxel in chemotherapy by its high LD$_{50}$ value, negligible toxicity and potent efficacy in carcinoma regression in vivo. These results suggest TPGS is an efficient emulsifier for NP-PTX preparation, and NP-PTX is a potential alternative delivery system for advanced ovarian cancer treatment.

Multi-step process is commonly required to synthesize such particles in existing strategies, which usually results in inherently inefficient delivery systems. We here engineered a simple, scalable, efficient and highly controllable system using a well-defined and predictable strategy. There were several advantages to deliver paclitaxel using this particulate system: 1) it avoids the use of toxic polyethoxylated castor oil; 2) it evades PVA and other repeated deletion of emulsifiers; 3) it can be orally or systemically administered; 4) it inhibits tumor growth as well as free paclitaxel; and 5) it maintains the release of paclitaxel at therapeutic concentration meanwhile significantly reduces the side effects.

Gao et al. reported that the paclitaxel encapsulation efficiency is 75.56% and loading efficiency is 5.35% in PLGA-mPEG NPs. They concluded that the formulated NPs inhibits...
HepG-2 cell growth the same as free paclitaxel injection. Previous studies have also reported equivalent drug loading efficiency of paclitaxel in NPs prepared with biodegradable polymers, including PLGA and PLGA-mPEG.\(^{13,41}\) In our present study, the drug encapsulation and loading efficiency were 92.9% and 19.8%, respectively, which are both higher than the previous studies. The main reason for these improvements was attributed to the high partition coefficient and retention in the TPGS applied to catalyze NPs formation. TPGS has been found as an excellent emulsifier that can align at the oil–water interface of to enhance the integrity and stability of the nanoparticle by lowering its surface energy at low concentrations,\(^ {42}\) which can prevent the diffusion of encapsulated paclitaxel from NP-PTX.

In summary, our results show that PLGA-mPEG NPs formulation has tremendous advantages versus original free drug in chemotherapy. NPs demonstrated 10-fold less toxicity which causes no organ damage. Meanwhile, nanoparticulate formation did not hinder the cytotoxic activity of paclitaxel \textit{in vitro} or the tumor growth inhibition \textit{in vivo}. Taken together, our newly developed paclitaxel-loaded NPs have wide broad application prospects in clinical chemotherapy.

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Conflict of Interest The authors declare no conflict of interest.

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