Encapsulated paclitaxel nanoparticles exhibit enhanced anti-tumor efficacy in A549 non-small lung cancer cells

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Abstract

In the present study, paclitaxel (PTX) were encapsulated with polyethylene glycol (PEG)-polylactide (PLA)/D-α tocopheryl polyethylene glycol 1000 succinate (TPGS) (PEG-PLA/TPGS) and the enhanced anti-tumor activity of this PTX mixed micelles (PTX-MM) was evaluated in lung cancer cells. The PTX-MM prepared by a solvent evaporation method was demonstrated to have high drug-loading efficiency (23.2%), high encapsulation efficiency (76.4%), and small size (59 nm). In vitro release assay showed the slow release behavior of PTX-MM, suggesting the good stability of the PTX-MM essential for long circulation time. In vitro kinetics assay demonstrated that PTX-MM could promote absorption and increase relative bioavailability. The anti-cancer efficiency of PTX-MM was also examined by both in vitro and in vivo studies. PTX-MM exhibits obvious cytotoxicity against lung cancer cells with much lower IC50 value when compared with commercial formulated PTX or PTX + TPGS. The xenograft tumor model studies on nude mice indicated that PTX-MM inhibits tumor growth more effectively than other formulations. It was also found that most of mixed micelles were integral in tumor site to exhibit anti-cancer activity. Our results suggested that the use of PTX-MM as an anti-cancer drug may be an effective approach to treat lung cancer.

Key words: lung cancer, nanoparticle, paclitaxel (PTX)

Introduction

Lung cancer is one of the most common cancers which is a leading cause of mortality worldwide [1]. Approximately 80%–85% of the newly diagnosed cases of lung cancer are non-small cell lung cancer (NSCLC). The majority of lung cancer cases are diagnosed within the last five years and it is still an unresectable and/or non-curable disease [2]. Till now, the efficient therapy for advanced-stage NSCLC is chemotherapy, and paclitaxel (PTX) is used as a first-line chemotherapeutic drug in NSCLC therapy [3,4].

As an antineoplastic agent that belongs to the taxane family, PTX was proved to accelerate microtubule assembly and inhibit signal transduction [5,6]. PTX was proved to exhibit anti-tumor activity by inducing DNA damage and cell death [7,8]. However, the poor aqueous solubility of PTX limited its anti-cancer efficacy. To improve
the aqueous solubility of PTX, the commercial formulation such as Taxol® and the various drug delivery systems including nanoparticles, liposomes, micelles, and other formulation have been developed [9–15]. However, due to the serious side effects or the lack of high anti-cancer efficiency, novel drug delivery systems still need to be developed.

Polymeric micelle-like particles have been extensively studied as delivery systems for different therapeutics. Polymeric micelle-like particles allow long circulation time and reduce opsonization and detection by macrophages [16,17]. Moreover, their small size and long circulation time favor their passive accumulation within tumor cells [16]. They are less likely to cause hypersensitivity than stealth liposomes while achieving greater penetration of solid tumors [17]. Polymeric micelle-like particles composed of polyethylene glycol (PEG) and polylactide (PLA) in di- and triblock copolymers have been used as delivery systems for chemotherapeutic drugs with high entrapment efficiency (EE) and small particle size. Among the numerous types of materials used for micelles formulation, those containing D-α-tocopheryl polyethylene glycol 1000 succinate (TPGS) conjugated with hydrophobic polymers have received more attention [18,19]. TPGS presents several valuable properties such as the ability to increase the solubility and permeability of drugs to across barriers [20]. TPGS also has the intrinsic capacity to inhibit P-glycoprotein (P-gp), a transmembrane efflux pump which has a major influence on cancer cells drug resistance [21,22]. Recent studies also showed the ability of TPGS-based nano-delivery systems to simultaneously co-deliver different chemotherapeutic drugs [23,24].

In the present study, PTX was encapsulated in PEG-PLA/TPGS micelles and tested for the first time. Our findings demonstrated that PEG-PLA/TPGS micelles are capable of encapsulating PTX with high efficiency. Moreover, the administration of PTX-loaded micelles induced an improved cytotoxic effect in lung cancer cells, reflecting a better therapeutic outcome. Our findings suggested that this approach could be potentially used in NSCLC treatment.

Materials and Methods

Cell lines and chemicals

Sensitive human alveolar adenocarcinoma cell line (A549) and multidrug-resistant cell line (A549/PTX) were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were grown in RPMI 1640 medium (Gibco, New York, USA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 U/ml streptomycin. The cells were cultured in an incubator (Thermo Electron Corporation, Waltham, USA) at 37°C under 5% CO2, PEG-PLA the average molecular weight of PEG and PLA were 4.2 and 2.1 kDa, respectively, and the polydispersity index was 1.05 was purchased from Yarebio (Shanghai, China). TPGS was purchased from Ningbo Dahongying Bio-Engineering Co., Ltd (Ningbo, China). PTX was purchased from Jiangsu Yew Pharmaceutical Co., Ltd (Wuxi, China). Verapamil was purchased from Hengrui Pharmaceutical Co., Ltd (Lianyungang, China). Tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was purchased from Amresco (Solon, USA).

Preparation and characterization of PTX-loaded mixed micelle

PTX-loaded mixed micelles (PTX-MM) were prepared by a solvent evaporation method. Briefly, in a 5-ml round-bottom flask, PTX (1 mg) and copolymers (up to 5 mg, PEG-PLA/TPGS = 2.5/1, w/w) were dissolved in 1 ml chloroform. Then chloroform was removed under vacuum at the room temperature to form a homogenous thin drug-polymer film. Next, the film was hydrated with 10 mm 4-((2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES)-buffered saline (HBS, pH 7.4) and sonicated for 10 min at room temperature. The resulting mixture was then centrifuged at 12,000 g for 10 min to yield clear micelle dispersion. The size, dispersity, and zeta potential of micelles were analyzed by zeta potential measurement with submicrometer particle size analyzer (Brookhaven Instruments Corp., Austin, USA). The drug-loaded polymeric micelles were dissolved in absolute ethyl alcohol. The drug loading (DL) and EE were determined by reverse-phase high performance liquid chromatography (RP-HPLC) with ultraviolet (UV) detection at 227 nm and calculated by equation (1) and equation (2), respectively.

\[
DL\% = \frac{\text{Amount of PTX in micelles}}{\text{Amount of feeding PTX-MM}} \times 100\% \quad (1)
\]

\[
EE\% = \frac{\text{Amount of PTX in micelles}}{\text{Amount of feeding PTX}} \times 100\% \quad (2)
\]

Transmission electron microscopy (TEM) and fourier transform infrared spectroscopy (FTIR)

PTX-MM samples were dropped onto 80-mesh film-coated copper grids. The grids were negatively stained at room temperature with 1% (w/v) phosphotungstic acid solution and dried at room temperature. The morphology of PTX-MM was observed by TEM. Molecular structure of synthesized copolymer was characterized by FTIR using KBr pellets in the wave number region of 400–4000 cm⁻¹.

In vitro release behavior of PTX-MM

To assess the release kinetics, dialysis cassettes (molecular weight cut-off 10,000 Da) were filled with 1 ml of PTX-MM (100 μg/ml) and immersed in 400 ml of different medium: dialysis buffer (PBS, pH 7.4 containing 0.03% Tween 80). At designated time points, aliquots of 2 ml of buffer were sampled and the same volume of fresh dialysis buffer was added to maintain the same dialysis condition. PTX was used as the control.

Kinetics of cellular uptake assay

A549 and A549/PTX cells were seeded at a density of 1 × 10⁵ cells/ml in a 24-well cell culture plate and incubated in the culture medium until they reached 80% confluence. The culture medium was carefully replaced by different formulations containing the same amount of PTX (100 μg/ml) at 37°C. The formulations were as follows: (i) PTX; (ii) PTX + TPGS (0.08%, w/v); and (iii) PTX-MM. At predetermined incubation time intervals, the cells were washed with cold PBS three times and the amount of PTX in cells was assayed by HPLC.

In vitro anti-tumor activity

The in vitro anti-tumor activity of PTX, PTX + TPGS, and PTX-MM were determined by a standard MTT assay. Briefly, A549 and A549/PTX cells were seeded in 96-well plates at a density of 1 × 10³ cells/well and cultured for 24 h. Then 180 μl of PTX formulations with concentration from 0.001 to 10 μg/ml were added, and cells were further cultured for 48 or 72 h. At the end of incubation, 20 μl of 5 mg/ml MTT (dissolved in PBS) was added to each well and the cells were stained at 37°C for 4 h. The medium then washed out by PBS and the cells were mixed with 150 μl of dimethyl sulfoxide (DMSO).
The absorbance was measured at 570 nm by microplate reader (Thermo Scientific, Waltham, USA). Relative cell viability (R\%) was calculated as follows: \[ R\% = \frac{A_{\text{test}}}{A_{\text{control}}} \times 100\% \]. The values were expressed as relative cell viability and IC50 values were calculated using nonlinear regression analysis.

**In vivo anti-tumor efficacy**

*In vivo* study was performed in 5–6 weeks old nude mice (BALB/c mice) that were provided by the Laboratory Animal Center of Zhejiang University (Hangzhou, China). All the animals were pathogen free and allowed to access food and water freely. The experiments were carried out in compliance with Animal Care and Use Committee of Institute of Biophysics guidelines. To establish human lung cancer xenografts, the mice were subcutaneously inoculated in the back with \(1 \times 10^6\) A549 or A549/PTX cells suspended in 20 μl PBS. Tumor-bearing mice were used 3 days post-tumor inoculation in experiments, at which point tumor volumes reached around 0.1 cm³. For *in vivo* tumor inhibition test, the A549/PTX tumor-bearing nude mice were randomly divided into four groups. Saline (control), PTX, PTX + TPGS (0.08%, w/v), or PTX-MM in saline was injected i.v. through a tail vein at days 3, 5, 7, 9, and 11. The injection dose of all PTX formulations was 20 mg/kg, and the injection volume of saline was approximately 0.1 ml/10 g body weight. Tumor volumes were monitored every two days. Tumor volume (V) was determined by measuring length (L) and width (W) with a caliper, and calculated as: \[ V = \frac{L \times W^2}{2} \]. At the end of the 30-day treatment, five mice from each treatment group were sacrificed by cervical dislocation, and the tumor mass was weighted and images of tumor from each group were also captured. The remaining 10 tumor-bearing mice in each group were used in the survival study.

**Intratumoral accumulation and localization of PTX-MM**

Fluorescence resonance energy transfer (FRET) occurs when a donor dye and an acceptor dye present within the range of Förster distance; the emission fluorescence from the excited donor dye is used as the excitation energy for the acceptor dye, resulting in emission of the acceptor fluorescence [25]. To observe the location of the mixed micelles at the tumor site, nile red (Nr) (acceptor), and coumarin-6 (C-6) (donor) were co-encapsulated with PTX in the mixed micelles to prepare dual-fluorescent-labeled PTX-MM, whereas confocal laser scanning electron microscopy (CLSM) and FRET analysis confirmed the integrity of mixed micelle in tumor site. Briefly, Nr, C-6, PTX, and copolymers were dissolved in 1 ml chloroform, and the other procedure for the preparation of C-6 and Nr-labeled PTX-MM (C-6/Nr-MM) was similar to that for the preparation of PTX-MM. C-6/Nr-MM was intravenously administrated to the A549/PTX tumor-bearing mice. Tumor was collected after 8 h of administration, washed with PBS, treated by freezing microtomy, and visualized by CLSM (Leica TCS SF5; Leica, Wetzlar, Germany). Hoechst 33258 was used for nuclear counterstaining.

**Table 1. The physicochemical characteristics of PTX-MM**

<table>
<thead>
<tr>
<th>Samples</th>
<th>DL (%)</th>
<th>EE (%)</th>
<th>Particle size (nm)</th>
<th>PDI</th>
<th>Zeta potential (mV)</th>
</tr>
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<tr>
<td>PTX-MM</td>
<td>23.2 ± 1.3</td>
<td>76.4 ± 5.2</td>
<td>58.9 ± 1.6</td>
<td>0.12 ± 0.01</td>
<td>-2.7 ± 1.5</td>
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PTX-MM, Paclitaxel loaded PEG-PLA/TPGS mixed micelle; DL, drug loading; EE, entrapment efficiency; PDI, polydisperse index. \( n = 3 \).

**Statistical analysis**

Data are expressed as the mean±standard deviation, and statistical analysis was performed by SPSS. The differences among groups were analyzed by one-way ANOVA followed by Bonferroni’s multiple comparison test or t-test, as appropriate. Differences were considered significant when \( P < 0.05 \).
Results

Preparation and characterization of PTX-MM

The physicochemical characterization and DL parameters of PTX-MM such as particle size, zeta potential, and PTX encapsulation were summarized in Table 1. The particle size and zeta potential are important indices for micelles. PTX were able to readily self-assemble to form nanomicelles with a particle diameter around 59 nm as measured, and the micelles solution was negatively charged with a mean zeta potential of approximately $-2.7 \pm 1.5$ mV. The PTX-MM solution showed good stability with high absolute value of the zeta potential. The stability of the micelles for oral administration was also demonstrated with small particle sizes and high zeta potentials. DL and EE are important indices to determine whether or not the PTX is suitable for preparation into the micelles. The DL of PTX-MM was 23.2 ± 1.3, and EE of PTX-MM was 76.4 ± 5.2. According to the particle size and DL value, PTX-MM is suitable for the subsequent studies. Physicochemical characteristic of nanoparticles was investigated by FTIR (Fig. 1C) and the formation of polymeric micelles loaded with PTX was validated.

Figure 3. Kinetic study of cellular uptake of PTX, PTX + TPGS, and PTX-MM (A) A549 cells, (B) A549/PTX cells. Each point represents the mean±SD ($n$ = 3).

Figure 4. In vitro anti-tumor activity of PTX, PTX + TPGS, and PTX-MM in A549 and A549/PTX cells. A549 and A549/PTX cells were incubated with different formulations for 48 h (A,B) and 72 h (C,D). Each point represents the mean±SD ($n$ = 9).
In vitro release of PTX-MM
The TEM images of the PTX-MM showed that the PTX micelles are spherical and homogeneous in aqueous solution (Fig. 1A,B). The particle size observed by TEM correlated well with the results measured by submicrometer particle size analyzer (Table 1), indicating that the polymeric micelles were well-dispersed in aqueous solution. Figure 2 exhibited the release profiles of PTX-MM and PTX, respectively. During the first 24 h, PTX released rapidly, while PTX-MM showed little release. After 144 h, 40% of the incorporated drug was still in the mixed micelles. The slow release behavior confirmed the stability of the PTX-MM, which ensures long circulation time after administration.

In vitro cellular uptake of PTX-MM
Then the cellular uptake of PTX-MM was investigated. Cellular uptake of different PTX formulations was examined in both A549 and A549/PTX cells. PTX and PTX + TPGS micelles were used as controls. PTX-MM was found to have greater cellular uptake than PTX or PTX + TPGS in A549 (Fig. 3A) and A549/PTX cells (Fig. 3B), which allowed PTX-MM treatment to be effective with a relatively small dose and without causing severe adverse effects. It also suggested that PTX-MM was a novel mixed micelles that could promote absorption and increase relative bioavailability.

In vitro cytotoxicity of PTX-MM on cancer cells
The anti-proliferative effect of various PTX formulations on A549 or A549/PTX cells was shown in Fig. 4. Cells were treated with different PTX formulations at different concentrations. The cytotoxicity was measured by MTT assay after 48 h or 72 h treatments. All three PTX formulations inhibited the tumor cell growth in a concentration- and time-dependent manner. As shown in Table 2, The IC50 of

<table>
<thead>
<tr>
<th>Formulation</th>
<th>A549 cells</th>
<th>A549/PTX cells</th>
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<tr>
<td></td>
<td>IC50 (48 h) (μg/ml)</td>
<td>IC50 (72 h) (μg/ml)</td>
</tr>
<tr>
<td>PTX</td>
<td>0.54 ± 0.03</td>
<td>0.13 ± 0.03</td>
</tr>
<tr>
<td>PTX+TPGS</td>
<td>0.51 ± 0.02</td>
<td>0.08 ± 0.02</td>
</tr>
<tr>
<td>PTX-MM</td>
<td>0.15 ± 0.03</td>
<td>0.04 ± 0.01</td>
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RRI = IC50 (PTX)/IC50 (PTX + TPGS) or IC50 (PTX)/IC50 (PTX-MM). Significant differences between PTX and PTX + TPGS group are indicated as *P < 0.05; between PTX and PTX-MM group are indicated as **P < 0.01; between PTX + TPGS and PTX-MM group are indicated as ***P < 0.01.

Figure 5. In vivo anti-tumor efficacy of saline, PTX, PTX+ TPGS, and PTX-MM in A549/PTX tumor-bearing nude mice (A) Kaplan–Meier curves showing the survival of A549/PTX tumor-bearing mice treated with different formulations (n = 10). (B) Tumor volume changes during 30-day treatments, each point represents the mean±SD (n = 5). (C) Tumor tissue weight after 30-day treatments. Each point represents the mean±SD (n = 5). (D) Tumor images of sacrificed mice after saline, PTX, PTX + TPGS, and PTX-MM treatments. Significant differences between groups are indicated as *P < 0.05, **P < 0.01.
PTX-MM was much lower when compared with PTX or PTX + TPGS with 48 h or 72 h of treatment time in A549 or A549/PTX cells. Thus, PTX-MM showed higher levels of cytotoxicity to cancer cells when compared with PTX or PTX + TPGS in both A549 and A549/PTX cells.

In vivo anti-tumor efficacy of PTX-MM
PTX-MM treatment in vivo showed a longer overall survival in tumor-bearing nude mice when compared with treatment with other formulations. There were no significant changes among groups treated with saline, PTX, or PTX + TPGS (Fig. 5A). The tumors in saline-treated group showed a fast and stable growth. The tumors in PTX and PTX + TPGS-treated groups showed a relatively high tumor inhibition, while those in PTX-MM showed the highest tumor inhibition (Fig. 5B). Consequently, the tumor weight in PTX-MM-treated group was much lower when compared with those in the control groups (Fig. 5C,D). The intratumoral accumulation and localization of PTX-MM in the above-mentioned xenografted tumors were examined by using C-6/Nr-MM and the results were shown in Fig. 6A. The micelles formed by the mixtures of the two copolymers were confirmed by FRET detection (Fig. 6B). Because the red fluorescence of Nr could be detected with excitation at 458 nm, indicating that FRET occurred. Our results showed that most of mixed micelles were integral in tumor site (Fig. 6C).

Discussion
In this study, the novel PTX-MM micelles were successfully synthesized for lung cancer chemotherapy. The PTX-MM was prepared by a solvent evaporation method. The size of the PTX-MM was about 59 nm. The drug-loading content and encapsulation efficiency of PTX-MM were ideal. In vitro release of PTX-MM was much slower when compared with the commercial formulated PTX. PTX-MM was found to have greater cellular uptake than PTX or PTX + TPGS in A549 and A549/PTX cells, which allowed PTX-MM treatment to be effective with a relatively low dose and with less severe adverse effects. Assessment of cytotoxicity suggested that the PTX-MM had higher cytotoxicity against A-549 cells A549 or A549/PTX cells than PTX or PTX + TPGS. Previous studies using similar nanoparticles showed that PTX-loaded copolymer PLA-TPGS-induced
apoptosis in HepG2 cells [26]. In addition, folate decorated PTX-loaded PLA-TPGS nanoparticles are more effective targeting at cancer cells both in vitro and in vivo than PTX alone [27]. A recent study demonstrated that TiO2, SiO2, and hydroxyapatite nanoparticles could strengthen intracellular tension and retard cellular migration [28]. Unfortunately, in the present study, we did not observe the effect of nanoparticles on cell viability. However, several studies using similar nanoparticles showed no cytotoxic effect when compared with the control [29,30].

A xenograft tumor model on nude mice indicated that PTX-MM inhibits tumor growth more effectively than other formulations, which may be attributed to the enhanced permeability and retention (EPR) effect of nanoparticles [31]. Previous studies indicated that iRGD-TPGS-conjugated PTX and shSur systems have higher anti-tumor efficacy due to its simultaneously exerting EPR effect and higher tumor-targeting efficiency [32]. A recent study also proposed the nanoparticle-induced endothelial leakiness (NanoEL) effect in TiO2-based nanomaterials, and the nanoparticle should be approximately 20 nm in size in order to cause NanoEL effect [33]. However, in the present study, the function of PTX-MM is unlikely via NanoEL effect, as the size of PTX-MM is around 60 nm. Finally, we found that most of mixed micelles were integral in tumor site, which is essential for its anti-cancer activity. All these results suggested that the PTX-MM developed in this study may be a potential drug delivery platform for the enhanced cancer therapy.

References