PEGylation of paclitaxel largely improves its safety and anti-tumor efficacy following pulmonary delivery in a mouse model of lung carcinoma

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Abstract

Pulmonary delivery offers an attractive route of administration for chemotherapeutic agents, with the advantages of high drug concentrations locally and low side effects systemically. However, fast clearance mechanisms result in short residence time of small molecule drugs in the lungs. Moreover, the local toxicity induced by antineoplastic drugs is considered a major obstacle for the clinical application of inhaled chemotherapy. In this study, we explored the utility of 6 kDa and 20 kDa polyethylene glycol-paclitaxel (PEG-PTX) conjugates to retain paclitaxel within the lungs, achieve its sustained release locally, and thereby, improve its efficacy and reduce its pulmonary toxicity. The conjugates increased the maximum tolerated dose of paclitaxel by up to 100-fold following intratracheal instillation in healthy mice. PEG-PTX conjugates induced lung inflammation. However, the inflammation was lower than that induced by an equivalent dose of the free drug and it was reversible. Conjugation of paclitaxel to both PEG sizes significantly enhanced its anti-tumor efficacy following intratracheal instillation of a single dose in a Lewis lung carcinoma model in mice. PEG-PTX 20k showed equivalent efficacy as PEG-PTX 6k delivered at a 2.5-fold higher dose, suggesting that the molecular weight of the conjugate plays a role in anti-cancer activity. PEG-PTX 20k conjugate presented a prolonged residency and a sustained paclitaxel release within the lungs. This study showed that PEGylation of paclitaxel offers a potential delivery system for inhalation with improved anti-cancer efficacy, prolonged exposure of lung-resident tumors to the antineoplastic drug and reduced local toxicity.

Key words: polymer-drug conjugates; inhaled chemotherapy; lung cancer; pulmonary delivery; paclitaxel; prodrug

Chemical compounds studied in this article

Polyethylene glycol (PubChem CID: 174); Paclitaxel (PubChem CID: 36314)

Abbreviations

BAL	Bronchoalveolar lavage
DLS	Dynamic light scattering
DMEM	Dulbecco's Modified Eagle Medium
PBS	phosphate-buffered saline
EPR	Enhanced permeability and retention
FBS	Fetal bovine serum
HBSS	Hank's balanced salt solution
HPLC	High Performance Liquid Chromatography
i.v.	Intravenous
i.t.	Intratracheal
LDH	Lactate dehydrogenase
LC-MS	Liquid chromatography mass spectrometry
MTD	Maximum tolerated dose
MW	Molecular weight
PEG	Polyethylene glycol
PEG-PTX	Polyethylene glycol-paclitaxel conjugate
PEG-PTX 6k	Polyethylene glycol-paclitaxel conjugate with PEG molecular weight of 6 kDa

PEG-PTX 20k	Polyethylene glycol-paclitaxel conjugate with PEG molecular weight of 20 kDa
PTX	Paclitaxel
SEM	Standard error of the mean

1. Introduction

Lung cancer is the leading cause of cancer-related death worldwide [1]. Chemotherapy is widely used to treat lung cancer either alone or, combined with surgical resection and radiotherapy [2]. However the 5-year survival rate only reaches 18% [3]. The poor efficacy of the treatments results from the late diagnosis and from the limited and nonspecific access of chemotherapeutics to lung tumors after intravenous administration [4, 5]. Pulmonary delivery is considered an attractive route of administration for chemotherapeutic agents, with the advantages of direct drug deposition at the diseased site and low systemic side effects. However, it is challenging to achieve sustained drug concentrations locally because of the efficient clearance mechanisms in the lungs. Small molecules are absorbed in the bloodstream within minutes and this results in a short residence time of chemotherapeutics in the lungs [6]. Moreover, safety issue remains a major consideration for inhaled chemotherapeutics. The inhalation of cytotoxic drugs may cause transient high local drug concentrations, which may lead to toxicity to the lung tissue [7-9]. Therefore, the benefits and the challenges of inhaled chemotherapy have stimulated interests in the development of delivery systems for retaining and progressively releasing anti-cancer drugs in the lungs [10-16].

Polymer-drug conjugates are prodrug systems with one or more drug molecules covalently conjugated to a hydrophilic polymer. They can offer sustained release of drugs and reduced toxicity. Polymer-drug conjugates of anti-cancer agents have been extensively studied following intravenous administration. The enhanced permeability and retention (EPR) effect favors the passive accumulation of anticancer agents into the tumor tissue when delivered intravenously [17]. In general, drugs are conjugated to polymers with biodegradable linkers, which allow the release of the active therapeutic. Conjugates containing ester or amide bonds

hydrolyze by non-specific esterase in extracellular environment or within cellular lysosomes, respectively.

As an alternative to inhalation of free drug formulations, polymer-drug conjugates would theoretically offer prolonged retention time of anticancer agents in the lungs and reduced toxicity to the lung tissue. In contrast to small molecules, macromolecules present long residence times in the airspaces [6]. However, the pulmonary delivery of polymer-drug conjugates in lung cancer has not been thoroughly investigated. Studies on the pulmonary administration of poly-L-glutamic acid paclitaxel conjugate (PGA-PTX) and doxorubicin-conjugated dendrimers have been reported [13, 14, 18]. These studies were performed in animal lung cancer models and showed that polymer-drug conjugates offer potential to improve tumor exposure to the cytotoxic drug and to provide better tolerance locally.

Hydrophilic polymers such as polyethylene glycol (PEG) improve the solubility of hydrophobic therapeutics but also increase retention time in the lungs. For instance, ester conjugates of prednisolone and 2 kDa PEG reduced prednisolone absorption rate across the pulmonary barrier by 8 fold following nebulization to the isolated perfused rat lung [19]. PEGs <2 kDa are effectively cleared from the lungs within 48 h. In contrast, PEG with large molecular weight (MW of 5 kDa and 20 kDa) can be retained in the lungs for up to 7 days [20]. This feature could favour the sustained release of drugs conjugated to large PEG in the lungs. Moreover, PEG is nontoxic and it can be eliminated by renal and hepatic pathways. It has already been approved for human use in dosage forms for intravenous, oral and pulmonary applications. In addition, conjugates have relatively high drug loading (10-30%) compared with nanocarrier systems [21]. There are various studies about PEGylated anti-cancer drugs by the intravenous route. PEGylated irinotecan, docetaxel, and SN38 (SN 38, 7-ethyl-10-hydroxy-camptothecin) are

under phase II or III clinical trials by the intravenous route for the treatment of solid tumors [22, 23]. However, the application of PEGylated chemotherapeutics in lung cancer using pulmonary delivery has not been investigated.

Paclitaxel (PTX) has been widely used to treat non-small cell lung cancer. The commercial product is Taxol®, which contains the water-insoluble paclitaxel formulated with Cremophor EL and dehydrated alcohol [24]. The treatment is currently administered by intravenous infusion in the clinic. However, significant adverse effects such as hypersensitivity reactions, hematologic, neuro toxicities have been reported and some are not only related to paclitaxel but also to Cremophor EL in the formulation [25, 26]. In addition, the oily nature of Cremophor will hamper its administration to the lungs. Therefore, developing drug delivery systems for inhaled paclitaxel without using Cremophor EL as solubilizer is needed.

This study explores the use of polyethylene glycol-paclitaxel (PEG-PTX) conjugates as a strategy to achieve a sustained release of anticancer agents in the lungs and thus, improve inhaled chemotherapy. The drug was conjugated to PEG with molecular weights of 6 kDa and 20 kDa as large PEG can be retained in the lungs over several days. PEG-PTX conjugates have previously been produced and characterized *in vitro* [27]. The conjugates were synthesized by "click" chemistry and contained hydrolysable ester bonds between PTX and PEG (Fig. 1). As the hydrolysis of the ester bonds by non-specific esterase proceeds, PTX is released in an active form. The *in vitro* data showed that PEG-PTX conjugates were able to slowly release PTX [27]. The conjugates have a half-life of more than 72 h in phosphate buffer saline and 7 to 9 h in bronchoalveolar lavage. The conjugates presented cytotoxicity to B16-F10 melanoma cells and LL/2 Lewis lung cancer cells in vitro, but less than Taxol® [27]. In this study, the antitumor

efficacy and safety of the conjugates are compared to those of free paclitaxel and Taxol® after pulmonary delivery in a murine model of lung carcinoma.

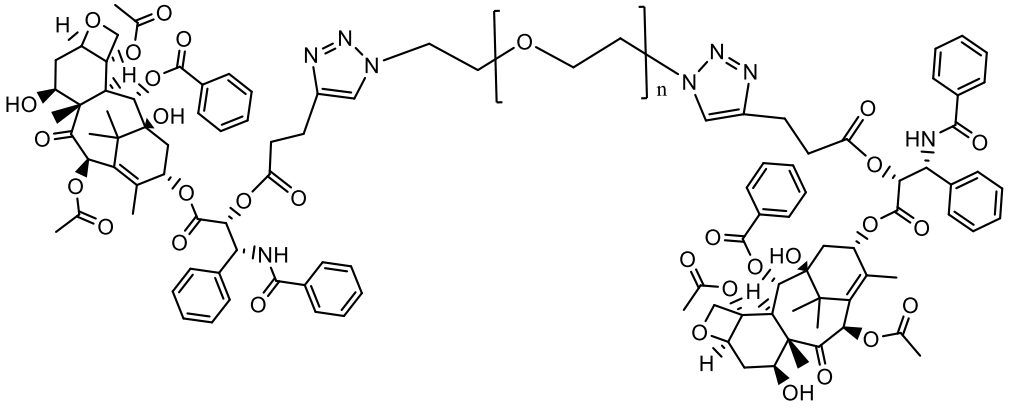

Fig. 1 The structure of PEG-PTX 6k and 20k conjugates. The PEG molecular weights were 6 kDa and 20 kDa. Paclitaxel was firstly modified with pentynoic acid and then conjugated to PEG-N_3 via 'click' chemistry. There were 1.8 PTX on 1 PEG molecule in both molecular weights of this structure [27].

2. Materials and methods

2.1 Materials

PTX was purchased from Chemieliva (Chongqing, China). PEG 6 kDa and 20 kDa were purchased from Iris Biotech (Marktredwitz, Germany). Taxol® was obtained from Brystol-Myers Squibb. Hanks balanced salt solution (HBSS), phosphate buffered saline (PBS), Dulbecco's modified Eagle's medium (DMEM), penicillin/streptomycin (Pen Strep), and fetal bovine serum (FBS) were obtained from Life Technologies (Belgium). Cell culture flasks and microplates were from Corning (Corning® T-75, Sigma-Aldrich, USA). Tissue-Tek® O.C.T. Compound and Cryomold were purchased from Sakura® Finetek (Torrance, CA). Superfrost TM microscope slides and blot were obtained from Gerhard Menzel B.V.&Co.KG (Braunschweig, Germany). Pierce micro BCA protein assay kit and Pierce lactate dehydrogenase (LDH) cytotoxicity assay kit were from Thermo Fisher Scientific (Leuven, Belgium). ONE-glo luciferase assay kit was purchased from Promega (Leiden, Netherlands).

HPLC grade acetonitrile was from Merck (Darmstadt, Germany). Ultrapure water was used throughout and all other reagents were of analytical grade.

2.2 PEG-PTX conjugates and Taxol®

PEG-PTX conjugates made of linear PEG 6 kDa or 20 kDa were prepared by conjugating paclitaxel to PEG at both its hydroxyl ends via click chemistry using azide linker triazole rings and ester bonds, as previously described [27]. The conjugates were white lyophilized powders and the solutions of conjugates were filtrated through 0.22 µm membrane before lyophilisation. 6k and 20k PEG-PTX conjugates were reconstituted in 37 °C sterile PBS according to the desired doses before administration to mice. Taxol® is the commercial formulation of paclitaxel, which is made of 6 mg paclitaxel, 50% of Cremophor EL and 50% of ethanol per mL. Taxol® was diluted with sterile PBS according to the desired doses and filtrated through 0.22 µm membrane before administration to mice.

2.3 Animals

Female C57BL/6NJR mice (8 to 10 weeks old, Janvier, Le Genest-StIsle, France) were kept on a 12-hour light-dark cycle and were allowed to food and water *ad libitum*. The experimental protocols were approved by the Institutional Animal Care and Use Committee of the Université catholique de Louvain (Permit number: 2012/UCL/MD/006). All studies were performed under anesthesia and all efforts were made to minimize animal suffering.

Mice were anesthetized by intraperitoneal injection of ketamine/xylazine (90/10 mg/kg) before receiving paclitaxel formulations by intratracheal instillation (i.t.). The mouse was fixed to make sure its neck was vertical. After gently pulling the tongue out, treatment solution was pipetted on the top of trachea. The tongue was released after two breaths were completed.

Single dose treatments of the different formulations were given in a volume of 50 µL per mouse. Blood samples were collected from the orbital sinus and kept at 4 °C overnight. After centrifugation at 12,000×g for 10 min, the serum was withdrawn and stored at -20 °C. Bronchoalveolar lavage (BAL) was performed after euthanizing the mice by cervical dislocation. One mL of HBSS was injected into the trachea and left for 15 s. 0.5 mL of the fluid was then withdrawn and re-injected into the lungs. All the BAL liquid was removed from the lungs afterwards. The volume of recovered BAL was recorded. The BAL samples were then centrifuged at 4,500×g for 10 min to remove the cells. The supernatants were collected and analyzed for total protein and LDH content immediately. . The lungs were then resected and kept in 0.5 mL HBSS on ice during the experiment. The lungs were homogenized and centrifuged at 3000×g rpm for 10 min. The supernatants were withdrawn and stored at -20 °C.

2.4 Maximum tolerated doses of PEG-PTX conjugates delivered intratracheally

To determine the toxicity of PEG-PTX conjugates, the maximum tolerated doses (MTD) of PEG-PTX conjugates and Taxol® were assessed following intratracheal instillation in female healthy C57BL/6NJR mice by the method describing in 2.3. PEG-PTX 6k conjugate was delivered by intratracheal instillation at doses of 10, 25, and 50 mg/kg (PTX equiv.). PEG-PTX 20k conjugate was delivered by intratracheal instillation at the dose of 20 mg/kg (PTX equiv.). Taxol® was delivered by intratracheal instillation at doses of 0.5, 1.2, 2, 5 mg/kg. Taxol® was also injected in the tail vein at doses of 10 mg/kg and 20 mg/kg in 200 µL. Mice were observed for 2 weeks. Symptoms and the numbers of surviving mice were recorded. The appropriate endpoints were defined based on signs of moderate severity, as described in the guidance from the Laboratory Animal Science Association. The MTD was defined as a maximum body weight loss of 20% and neither death, nor severe symptoms occurrence within 2 weeks.

2.5 Local toxicity of PEG-PTX conjugates in the lungs

The local toxicity of the PEG-PTX conjugates and Taxol® were assessed in healthy female C57BL/6NJR mice 24 hours, 72 hours and 7 days post intratracheal instillation. PEG-PTX 6k, 20k conjugates and Taxol® were delivered by intratracheal instillation in 50 μL at their MTDs, i.e., 50 mg/kg, 20 mg/kg, and 0.5 mg/kg, respectively. The local toxicity of PEG-N_3 6k was also investigated at the equivalent dose of 50 mg/kg PTX. In addition, a low dose of PEG-PTX 6k of 0.5 mg/kg and Cremophor EL at 0.5 mg/kg (PTX equiv.) were assessed 24 hours post intratracheal delivery. Mice were sacrificed at the predetermined time points and about 1 mL bronchoalveolar lavage was recovered and centrifuged immediately according to the procedures described in 2.3. Total protein and lactate dehydrogenase (LDH) in BAL supernatant were analyzed by Pierce BCA protein assay kit and LDH assay kit, respectively. Cell pellets were reconstituted at a concentration of 20,000 cells/mL and total live cells were counted by Türk's solution method (Merck KGaA, Darmstadt, Germany). The differential cell counts were obtained by cytocentrifugation and coloration with Diff Quick® (Medion Diagnostics AG, Switzerland). Macrophages, neutrophils and lymphocytes were counted under the microscope (×40 magnification) by counting 300 cells and recording cell numbers in each type. The percentages of each cell type were then calculated. The numbers of cell components were estimated by multiplying the total cell number with the percentages of each cell type.

The lungs were inflated with 1 mL Tissue Tek/PBS (1:1) and carefully removed from mice. The left lobes were embedded into Tissue Tek cryomatrix and then placed into liquid nitrogen to freeze rapidly. Frozen sections (10 μm) were performed with Cryostat (Leica Microsystems, Wetzlar, GE) and stained with haematoxylin-eosin (Sakura DRS 601). The sections were

examined by Leica slide scanner SCN 400 (Leica Biosystems, Wetzlar, GE).

2.6 Anti-tumor efficacy of PEG-PTX conjugates in Lewis lung carcinoma

Murine Lewis Lung Carcinoma cell line (LL/2-luc-M38 Bioware Cell Line, Caliper Life Sciences, Inc) stably expressing luciferase was a gift from Prof. Didier Cataldo, University of Liege (Belgium). LL/2-luc-M38 was cultured at 37 °C in 5% CO2, in DMEM supplemented with 10% FBS, 1 mM sodium pyruvate and 1% Pen Strep.

C57BL/6NJR female mice were randomly assigned to 5 groups according to treatments (n=6). A Lewis lung carcinoma model was established by tail vein injection of Lewis Lung carcinoma cells. On day 0, each C57BL/6NJR female mouse received 2×10^5 cells (passage 17 or 18) in 0.1 mL PBS intravenously (i.v.). On day 7, single dose treatments of PEG-PTX 6k, 20k conjugates and Taxol® were delivered to 3 groups of mice by intratracheal instillation in 50 μL at their MTD, i.e., 50 mg/kg, 20 mg/kg, and 0.5 mg/kg, respectively. The preparation of the conjugates and Taxol® solutions followed the procedure described in 2.2. 50 μL PBS was administered intratracheally to one group of mice as a control. In addition, one group of mice received 20 mg/kg of Taxol® in 200 μL by tail vein injection to compare the efficacy of different administration routes. Mice were observed for symptoms and body weights were recorded. On day 14, all mice were euthanized by cervical dislocation. The lungs were immediately taken. Lung weights were recorded. The number of lung metastatic cells were counted using bioluminescence on total resected and grounded lungs. One glo® luciferase assay was conducted to detect luciferase expression in the tumorous lungs. The assay contains a luciferase substrate, generates bioluminescence after conversion by tumor cells expressing luciferase. The bioluminescence was measured by a Victor Multilabel Plate Reader

(PerkinElmer, US). The number of lung tumor cells was calculated based on the calibration curve obtained with LL/2-luc-M38 cells cultured *in vitro.*

2.7 Kinetics of in vivo distribution in the respiratory tract

PEG-PTX 20k and Taxol® were investigated for their release kinetics and biodistribution in vivo. Female C57BL/6NJR mice were anesthetized by ketamine/xylazine (90/10 mg/kg) intraperitoneal injection. PEG-PTX 20k conjugates and Taxol® were delivered by intratracheal instillation in 50 µL at their MTD, i.e., 20 mg/kg and 0.5 mg/kg, respectively. Blood was taken immediately after administration of treatments. Mice were then euthanized. This time point was marked as 0h. 1 mL BAL and lungs were taken according to the procedure described in 2.3. Blood, BAL and lungs were also collected 24h and 48h post intratracheal instillation. To extract paclitaxel from serum, BAL and suspensions of homogenized lungs, acetonitrile was added at 1:1 (v/v) ratio to the samples and vortexed for 15 seconds. The suspensions were then centrifuged at 10,000 × g for 10 min and the supernatants were withdrawn. The supernatants were then dried under nitrogen flow at room temperature. The resulting residues from blood samples were reconstituted in 100 µL water/acetonitrile (1:1 v/v). The residues from BAL and lung samples were reconstituted in 300 µL water/acetonitrile (1:1 v/v). The samples were then centrifuged (10000 × g, 10 min) and 20 µL of the supernatant were analyzed by HPLC-MS (for PTX analysis) or by HPLC-UV (for analysis of the remaining PEG-PTX conjugates) by UV absorbance. The use of HPLC-UV for the analysis of PEG-PTX 20k was necessary because of the lack of proper signal for this large molecule with the LC-MS used in this study. The recovery after the extracting method was measured by adding PTX stock solution (200 µg/mL) to BAL, lungs and serum, respectively, to achieve a final concentration of 10 µg/mL PTX in these matrixes. These samples were then processed following the method

described above. The recovery was calculated as the percentage of detected PTX vs the amount added in. The recovery from BAL, lungs and serum at 10 µg/mL were 99.6%, 101.1% and 93.5%, respectively.

Paclitaxel was analyzed by LC-MS using an LTQ-Orbitrap mass spectrometer coupled to an Accela HPLC system (Thermo Fischer Scientific). Analytic separation was achieved using reverse phase C18 column (LiChrospher 100 RP-18 5 µm particles, 250 x 4 mm, Merck, Darmstadt, Germany). Mobile phases A and B consisted of H_2O / formic acid 99.9:0.1 (v/v) and acetonitrile. The gradient (1 mL/min) was performed as follows: starting at 40% B and reaching linearly 90% B in 13 min. This was followed by 7 min at 90% B before equilibrating at 40% B. An ESI source operated in the positive mode was used for the MS analysis. The ESI spray voltage was set at 5.0 kV and the capillary temperature at 275°C while the sheath gas flow and auxiliary gas flow were set at 20 and 10 arbitrary units, respectively. Paclitaxel was analyzed as the $[M+H]^+$ ion (m/z 854.33823). A standard curve was established in the range of 0.1-50 µg/mL of PTX in BAL following the extraction method above (correlation coefficient R= 0.9999, LOD = 0.13 µg/mL, LOQ = 0.44 µg/mL). Standard curves were also established in homogenized mouse lung suspension and mouse serum in the range of 0.1-10 µg/mL of PTX following the same extraction method above (R = 0.9948 and 0.9994).

HPLC-UV was carried out using the Hewlett Packard series 1100 system (Agilent Technologies, Palo Alto, CA) with a reverse phase C18 column (NUCLEOSIL® 300-5 C18 · 5 µm particles · 300 Å pores, 250mm×4.6mm, MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany). The mobile phase was acetonitrile and water eluted at 1 mL/min using a gradient protocol as follows: a linear gradient from acetonitrile 40% to 60% for 20 minutes, a linear gradient from acetonitrile

60% to 40% for 5 minutes. Absorbance of the column effluent was monitored at 227 nm. The areas under the peaks of conjugates were monitored.

2.8 Statistics

Results were shown as mean ± standard error of the mean (SEM). Mann-Whitney test was performed using the software GraphPad Prism to demonstrate statistical differences ($p < 0.05$) between groups.

3. Results

3.1 Conjugation to PEG increased the MTD of paclitaxel post-intratracheal delivery

An acute toxicity study was conducted to assess the MTD of the PEG-PTX conjugates following intratracheal instillation. The maximum doses tested were 50 mg/kg and 20 mg/kg for PEG-PTX 6k and 20k conjugate, respectively, because these doses reached the limits of their solubility in PBS (i.e., 145 mg/mL and 73 mg/mL for PEG-PTX conjugates 6k and 20k, respectively, or 30 mg/mL and 5.8 mg/mL PTX equivalent, respectively) [27]. As controls, the MTD of Taxol® was assessed following intratracheal instillation and intravenous injection.

All the intratracheal instillation groups showed body weight loss 1-2 days post-delivery (Fig. 2 A, B and C). A maximum of 7% of body weight loss was found in the group of PEG-PTX 6k 50 mg/kg (PTX equiv.) 1 day post-delivery. A maximum of 14% of body weight loss was found in the group of PEG-PTX 20k 20 mg/kg (PTX equiv.) at 2 days post-delivery. However, these weight losses were reversible. All mice from the conjugate groups and Taxol® 0.5 mg/kg group recovered their initial body weights within 3-4 days post-delivery.

Subdued behavior, lung noises and hunching were found in the groups of PEG-PTX 6k 50 mg/kg (PTX equiv.), PEG-PTX 20k 20 mg/kg (PTX equiv.) as well as in the group receiving Taxol® 0.5 mg/kg. However, these symptoms disappeared after 4-5 days of delivery. Death was found immediately after delivery in the group receiving Taxol® 5 mg/kg intratracheally. The mice receiving Taxol® 2 mg/kg and 1.2 mg/kg intratracheally survived for 1 day and 2 days post-delivery, respectively. Death was then found on the second and third days post-delivery. Mice were found to have tremors, lung noises, subdued behavior as well as hunching in the groups receiving Taxol® 1.2 mg/kg and above. The dead mice of the group receiving Taxol® 1.2 mg/kg were examined and lung haemorrhage was found. Therefore, the MTDs of PEG-PTX 6k and 20k following intratracheal instillation were 50 mg/kg (PTX equiv.) and 20 mg/kg (PTX equiv.), respectively (Table 1). These values were 100-fold and 40-fold the MTD of Taxol®, i.e., 0.5 mg/kg.

The MTD of Taxol® delivered by intravenous injection was also studied. Based on the reports in the literature [21, 28], two doses of 10 mg/kg and 20 mg/kg were delivered. Both groups of mice were found to have transient prostration for 5-10 minutes. The body weights of the 2 groups stably increased for 2 weeks (Fig. 2D). Therefore, the MTD of Taxol® following intravenous injection was 20 mg/kg in this study.

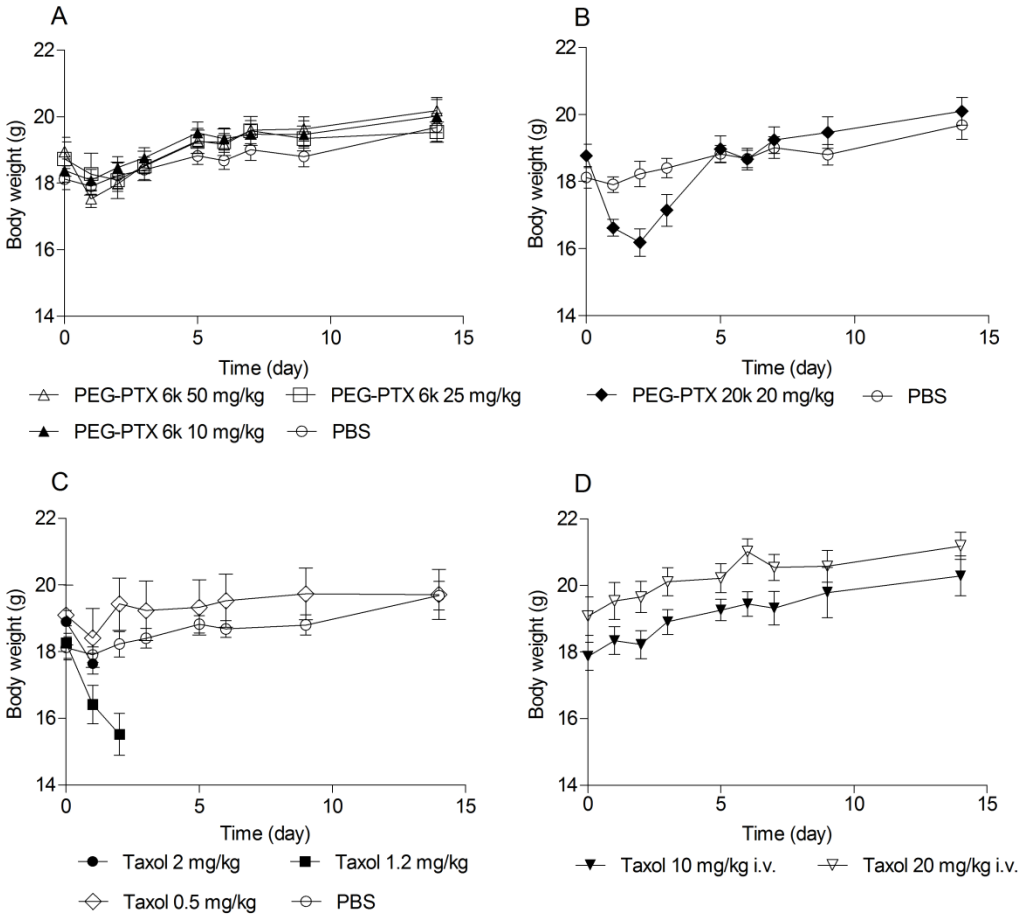

Fig. 2 Body weights of mice receiving PEG-PTX conjugates and Taxol® intratracheally and intravenously. (A) PEG-PTX 6k conjugate delivered intratracheally at different doses, (B) PEG-PTX 20k conjugate delivered intratracheally, (C) Taxol® delivered intratracheally at different doses, (D) Taxol® delivered intravenously at different doses. Mean ± SEM are shown, n=6 mice per group.

Table 1. MTD of PEG-paclitaxel conjugates and Taxol® post i.t. or i.v. injection.

	MTD (PTX equiv.)
PEG-PTX 6k i.t.	>50 mg/kg
PEG-PTX 20k i.t.	>20 mg/kg
Taxol® i.t.	0.5 mg/kg
Taxol® i.v.	20 mg/kg

n=6 mice per group

3.2 Local toxicity studies of PEG-PTX conjugates in the lungs

The local toxicity of the PEG-PTX conjugates to the lungs was assessed in healthy female C57BL/6NJR mice. Conjugates and Taxol® were administered at their MTDs. PEG-N_3 6k was the synthetic material of PEG-PTX 6k. It was tested to verify PEG toxicity to the lung tissue. We also tested Cremophor EL at the corresponding dose of 0.5 mg/kg PTX, which would give a better understanding of the pulmonary toxicity of Taxol®.

We analyzed biochemical inflammation markers in BAL in order to check if PEG-PTX induced inflammation in the lungs. Total protein was analyzed to detect the alteration of the alveolar-capillary barrier. Results showed that the groups of Taxol® 0.5 mg/kg, PEG-PTX 6k 50 mg/kg (PTX equiv.), PEG-PTX 20k 20 mg/kg (PTX equiv.) and Cremophor EL 0.5 mg/kg (PTX equiv.) induced large increases of total protein levels at 24h and 72h (Figs. 3A and 4A). However, PEG-PTX 6k at a lower dose of 0.5 mg/kg (PTX equiv., Fig. 4A) and PEG-N_3 6k 50 mg/kg (PTX equiv., Fig. 3A) did not induce an increase of total protein levels. At Day 7, the total protein concentrations decreased to the normal levels in the groups of Taxol®, PEG-PTX 6k and 20k (Fig. 3A).

The extracellular presence of lactate dehydrogenase (LDH), an intracellular enzyme, can reflect injury to pulmonary cells. Taxol® 0.5 mg/kg increased LDH levels in bronchoalveolar lavage 24h after i.t. delivery (Fig. 3B). Cremophor EL delivered i.t. at the corresponding dose of 0.5 mg/kg paclitaxel also showed a significant increase of LDH level (Fig. 4A). PEG-PTX 6k and 20k at their MTD caused significant increases of LDH in BAL 24h post i.t. delivery. However, PEG-N_3 6k delivered at the equiv. dose of PEG-PTX 6k 50 mg/kg (PTX equiv.) did not increase LDH level significantly. The LDH level of PEG-PTX 6k at a low dose of 0.5 mg/kg, which was the MTD of Taxol® i.t., remained at the same level as the control (Fig. 4A). At Day 7, the LDH

concentration decreased to the normal level in the groups of Taxol®, PEG-PTX 20k, and PEG-N_3 6k. The group of PEG-PTX 6k 50 mg/kg (PTX equiv.) showed decreased LDH concentration compared with 24h and 72h, but it still had higher LDH level than the PBS control group.

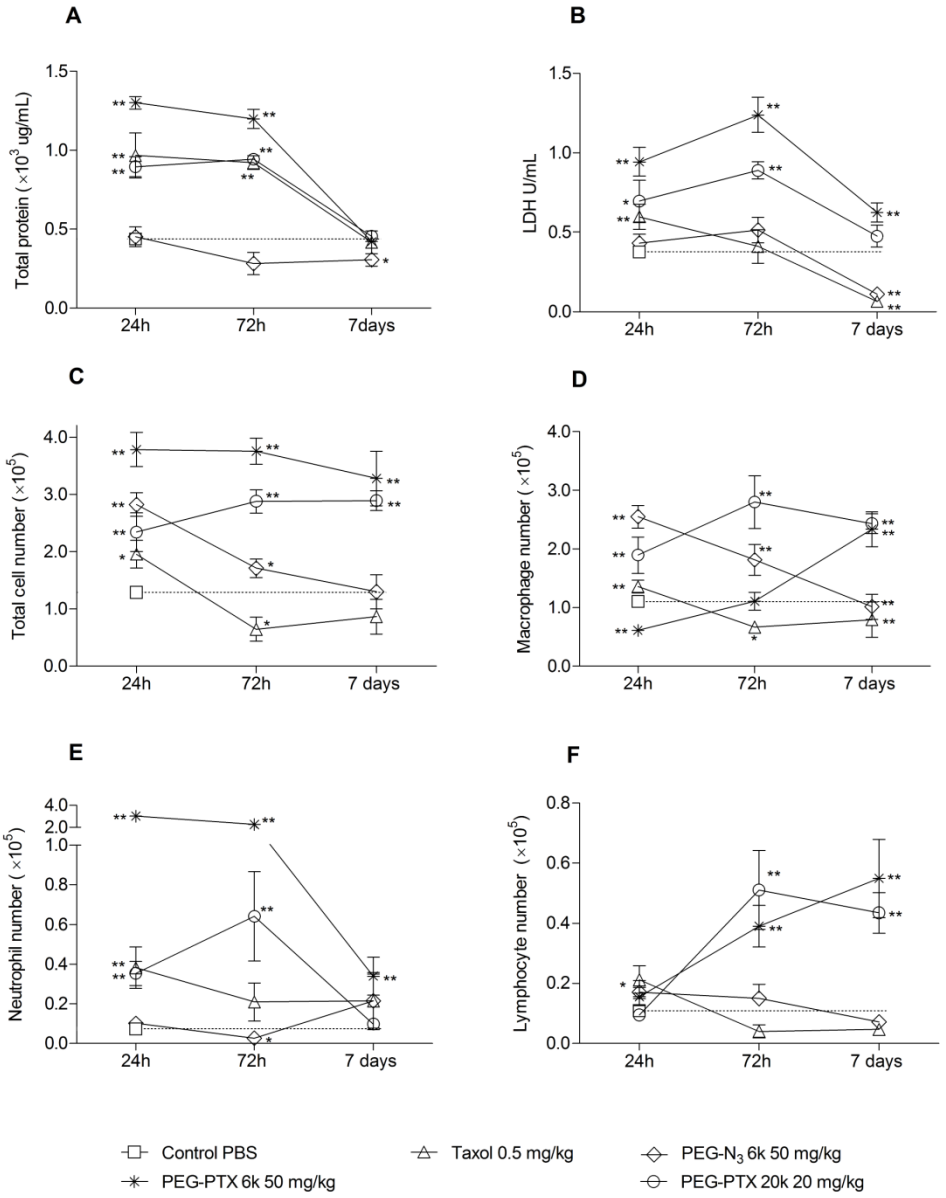

Fig. 3 Local toxicity of PEG-PTX conjugates, Taxol® and free PEG-N_3 6k. (A) Total protein (B) LDH level (C) total cell numbers (D) macrophage numbers (E) neutrophil numbers and (F) lymphocyte numbers in BAL at different time points post intratracheal instillation of PEG-PTX conjugates, Taxol®, and PEG-N_3 6k. The control PBS was only performed at 24h and extrapolated to day 7. Mean and SEM are shown, n=4-6 mice per group, * $p < 0.05$, ** $p < 0.01$, compared with control group (Mann-Whitney).

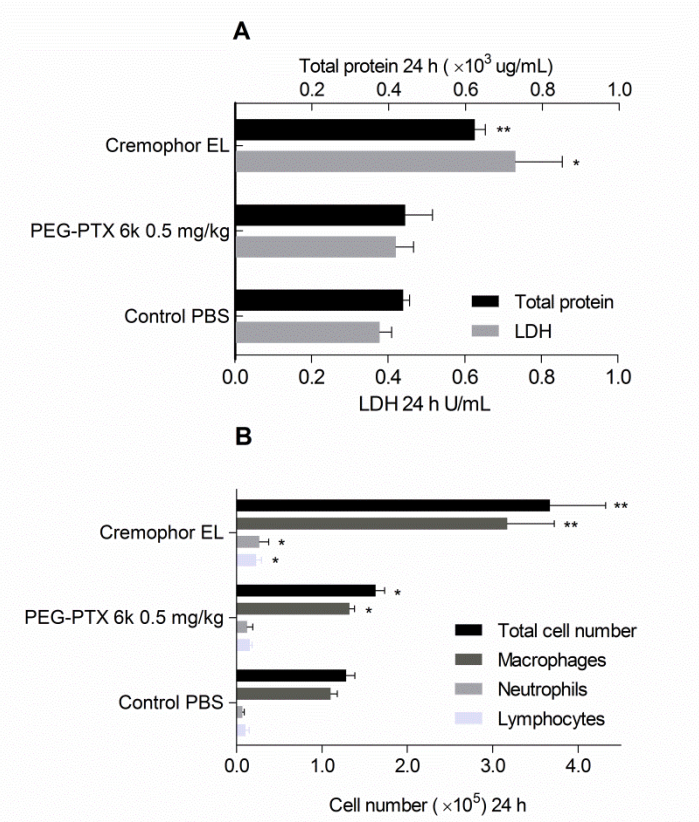

Fig. 4 Total protein, LDH level (A), and cell components (B) in BAL at 24h post intratracheal instillation of Cremophor EL, PEG-PTX 6k 0.5 mg/kg and PBS. Mean and SEM are shown, n=4-6 mice per group, * $p < 0.05$, ** $p < 0.01$, compared with control group (Mann-Whitney).

Total cells number and cell distributions in BAL fluid can provide an indication on the degree of pulmonary inflammation. There were large increases of total cell number in PEG-PTX 6k 50 mg/kg and 20k 20 mg/kg groups at 24h, 72h, and 7 days. The total cell number in the Taxol® group increased at 24h but decreased to normal level 72h post-delivery (Fig.3 C). The group of PEG-PTX 6k at the low dose of 0.5 mg/kg presented an increase of total cell number at 24h but less than the Taxol® group at the same dose (Fig. 4B). The group treated with only PEG-N_3 6k at the corresponding dose of 50 mg/kg PTX had an increase of total cell number at 24h, but the number decreased to normal level at day 7. Cremophor EL also induced a large increase of total cell number in BAL at 24h (Fig. 4B).

The number of neutrophils in BAL fluid is a cellular marker of inflammation. When PEG-PTX 6k

was administered at the high dose of 50 mg/kg (PTX equiv.), the neutrophil number rose significantly to more than 10-fold that of the control group at 24h and 72h, and decreased to normal level at day 7 (Fig. 3E). In the groups of PEG-PTX 20k and Taxol®, increases of neutrophil numbers were found at 24h and 72h, but neutrophil numbers were less than that of the PEG-PTX 6k group at all time points tested. The influx of neutrophils contributed to the increase of total cell numbers in these three groups. When PEG-PTX 6k was given at a low dose of 0.5 mg/kg, there was no significant increase of neutrophil number (Fig. 4B). PEG-N_3 6k 50 mg/kg (PTX equiv.) did not induce a significant increase of neutrophils at all time points tested. Cremophor EL given at the dose of 0.5 mg/kg (PTX equiv.) showed a significant increase of neutrophils numbers 24h post-delivery, but less than Taxol® (Fig. 4B).

The numbers of lymphocytes increased over time in the groups receiving high doses of PEG-PTX 6k and 20k, which indicated immune response might be induced by high dose of PTX (Fig. 3F). In addition, foamy macrophages were found in the groups receiving PEG-PTX 6k, 20k at their MTD and PEG-N_3 6k at 50 mg/kg (PTX equiv.) at all time points (Fig. 5).

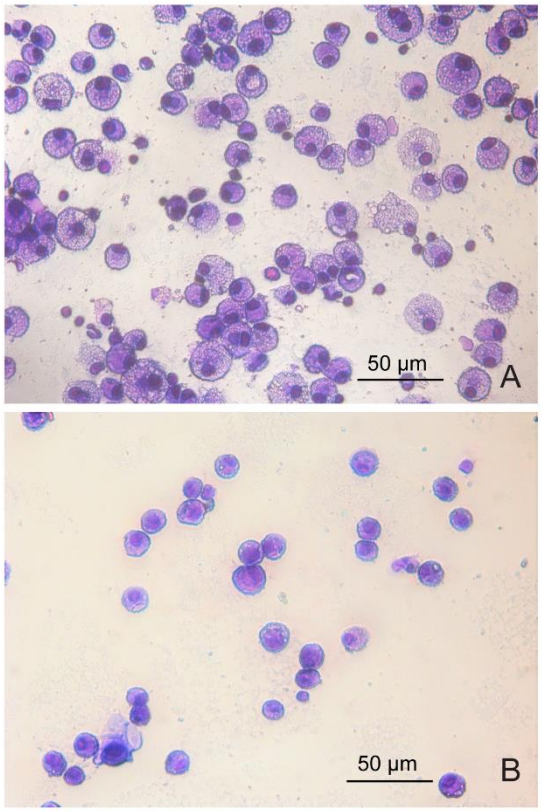

Fig. 5 Representative pictures showing foamy macrophages in (A) PEG-PTX 20k group 7 days post-delivery and their absence in (B) PBS control group (colored by Diff Quick®, magnification under optical microscope).

Generally, the histological examination showed no significant damages and changes in the structure and morphology of the lung parenchyma in all tested groups 24h post-delivery (Fig. 6). This was probably because obvious damage to the lung parenchyma had not been induced yet. The group receiving PEG-PTX 6k at a low dose of 0.5 mg/kg did not show obvious differences in morphology as compared with the PBS control group. There might be damages in the integrity of lung structure in some samples of Taxol® 0.5 mg/kg group when compared with the control. The thicknesses of bronchioles and alveolar epithelium appeared to be increased in groups treated with Taxol® 0.5 mg/kg, PEG-PTX 6k 50 mg/kg (PTX equiv.), PEG-PTX 20k 20mg/kg (PTX equiv.) and PEG-N_3 6k 50 mg/kg (PTX equiv.), compared with the negative control. This might reflect an over-production of mucus.

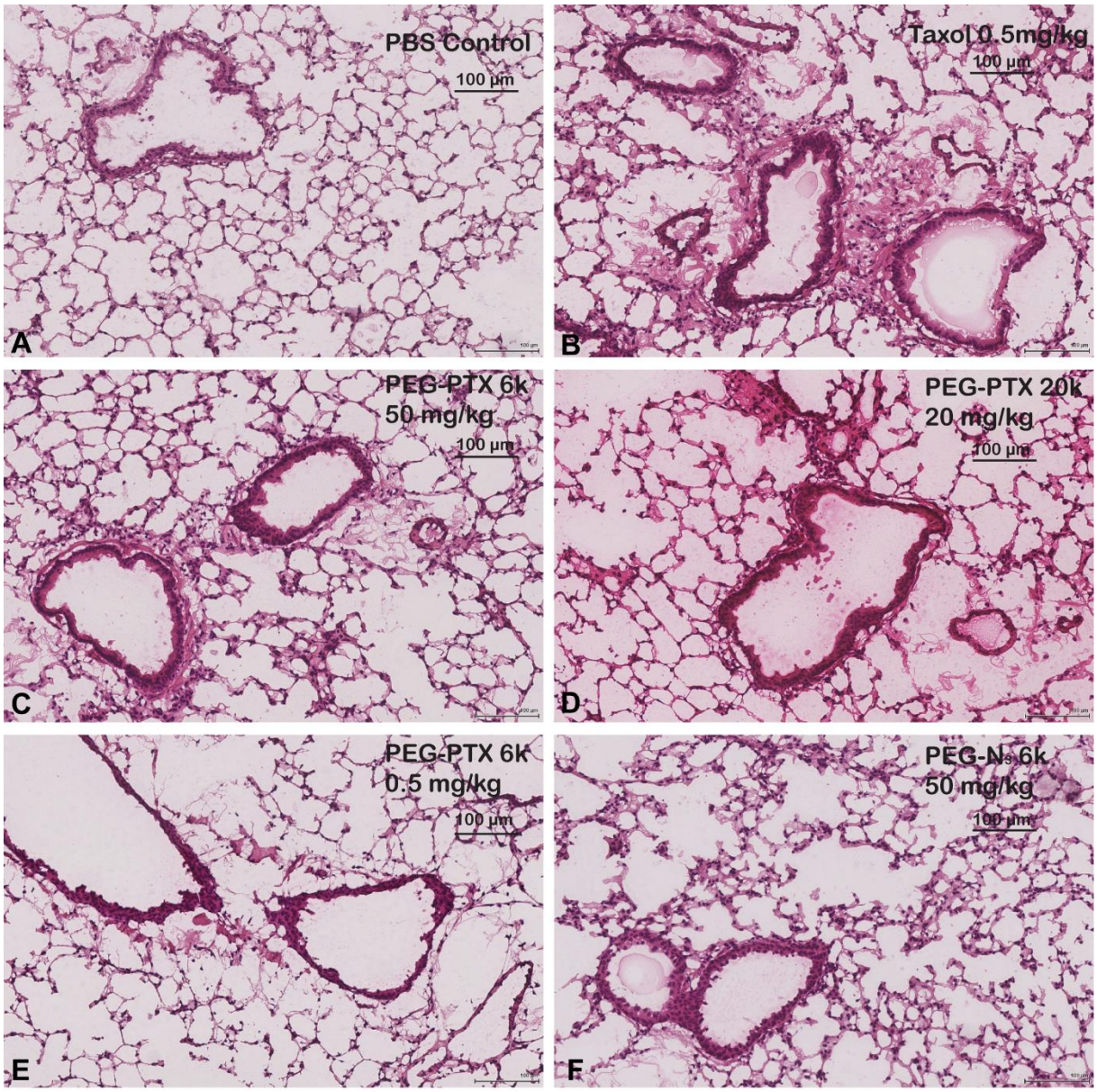

Fig. 6 Representative pictures showing lung morphology post intratracheal delivery of PBS control (A), Taxol® 0.5 mg/kg (B), PEG-PTX 6k 50 mg/kg (C), PEG-PTX 20k 20 mg/kg (D), PEG-PTX 0.5 mg/kg (E), and PEG-N_3 50 mg/kg (F) (Hematoxylin-Eosin staining).

3.3 PEG-PTX conjugates increased anti-tumor efficacy in a murine model of lung carcinoma

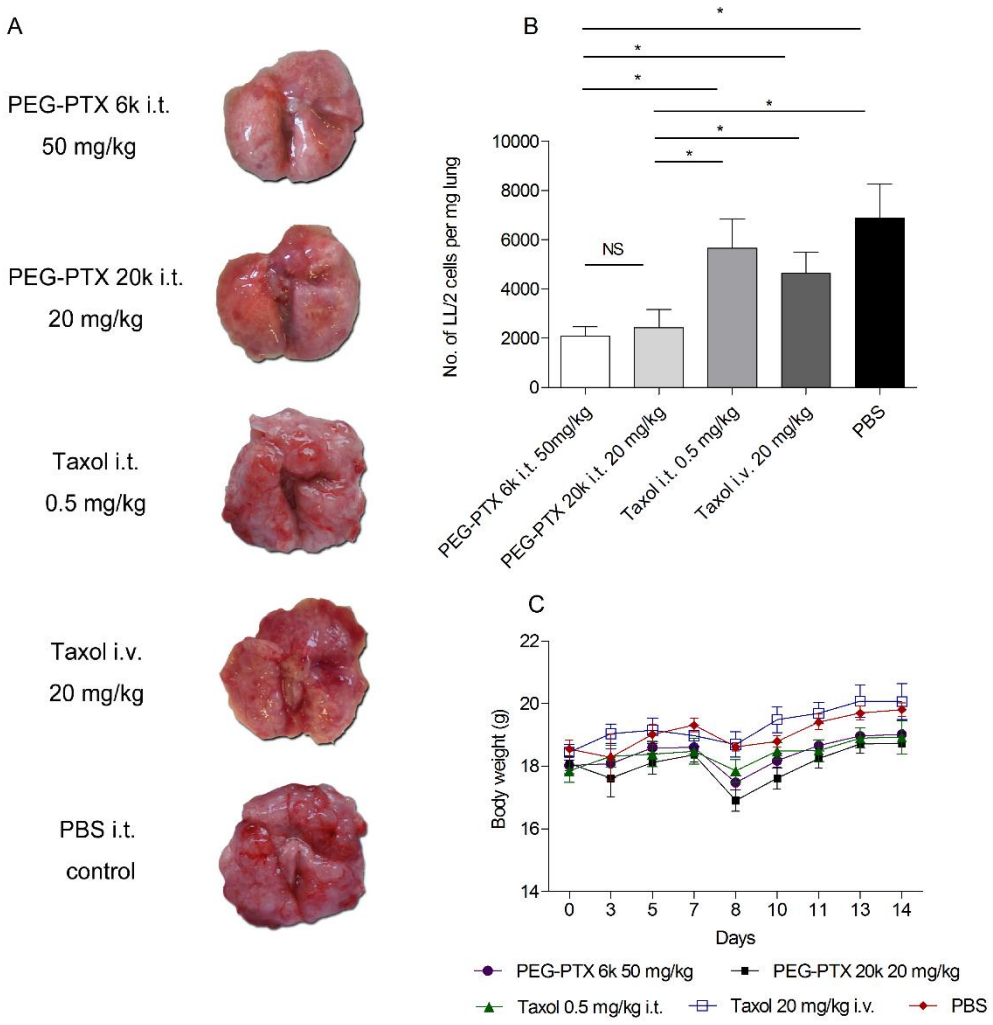

Fig. 7 Efficacy assessment of PEG-PTX conjugates and Taxol® delivered by intratracheal instillation or intravenous injection in a murine model of Lewis lung carcinoma. (A) Representative images of mouse lungs. (B) Numbers of LL/2 tumor cells per milligram lung tissue. (C) Body weights of mice. Mean ± SEM are given, n=6-7. * $p < 0.05$; NS, no significant difference (Mann-Whitney). Similar results were obtained in two independent experiments.

Both PEG-PTX 6k and 20k at their MTDs reduced the tumor cell number in the lungs compared to the non-treated control group (Fig. 7). When comparing with Taxol® 0.5 mg/kg i.t., PEG-PTX 6k and 20k also showed superior efficacy at doses of 50 mg/kg and 20 mg/kg, respectively. Taxol® delivered i.t. was not able to significantly reduce tumor cells in the lungs. There was no statistical difference between the groups of PEG-PTX 6k and 20k (Fig. 7B), which indicated that same anti-tumor efficacy could be achieved at a lower dose of paclitaxel with higher molecular

weight of PEG. Taxol® was also injected intravenously to compare the efficacy of different administration routes. However, intravenous Taxol® at its MTD was not able to significantly decrease tumor cell number in the lungs. There was a slight and reversible body weight loss 1 and 2 days post-intratracheal instillation in the conjugates and Taxol® groups (Fig. 7C).

3.4 PEG-PTX conjugates prolonged the retention time of paclitaxel in the lungs

PEG-PTX 20k (20 mg/kg PTX equiv.) presented equivalent anti-cancer efficacy and less local toxicity compared with PEG-PTX 6k at a 2.5 higher dose (50 mg/kg PTX equiv.). Therefore, we selected PEG-PTX 20k to investigate the release of PTX from the conjugate *in vivo* and we used Taxol® as a control. PEG-PTX 20k presented a prolonged release of PTX in both BAL and lungs (Fig. 8). Indeed, at 48h post-administration, PTX amount in BAL was still 40% of the dose recovered at time 0 (Fig. 8C). Most of the PTX released from the conjugate was present in the BAL at all time points, and only a small percentage was present in the lung tissue (Fig. 8C). In serum, the PTX released from the conjugate was below the limit of quantification at 24h and 48h post-administration. PEG-PTX 20k showed a long retention time in both BAL and lungs. 43% and 20% of PEG-PTX 20k initial dose remained in BAL and lungs 48h post-administraton, respectively (Fig. 8D). There was no PEG-PTX conjugate detected in serum at all time points. As a contrast, Taxol® delivered at the dose of 0.5 mg/kg (10 μg of PTX per mouse) showed a quick clearance from the respiratory tract. Only 3.8 μg and 0.6 μg of PTX were present in BAL and lungs, respectively, at time 0 (Fig. 8A&B), but in serum the concentration of PTX was below the limit of quantification. PTX was also below the limit of quantification in serum, BAL and lungs 24h and 48h post-delivery.

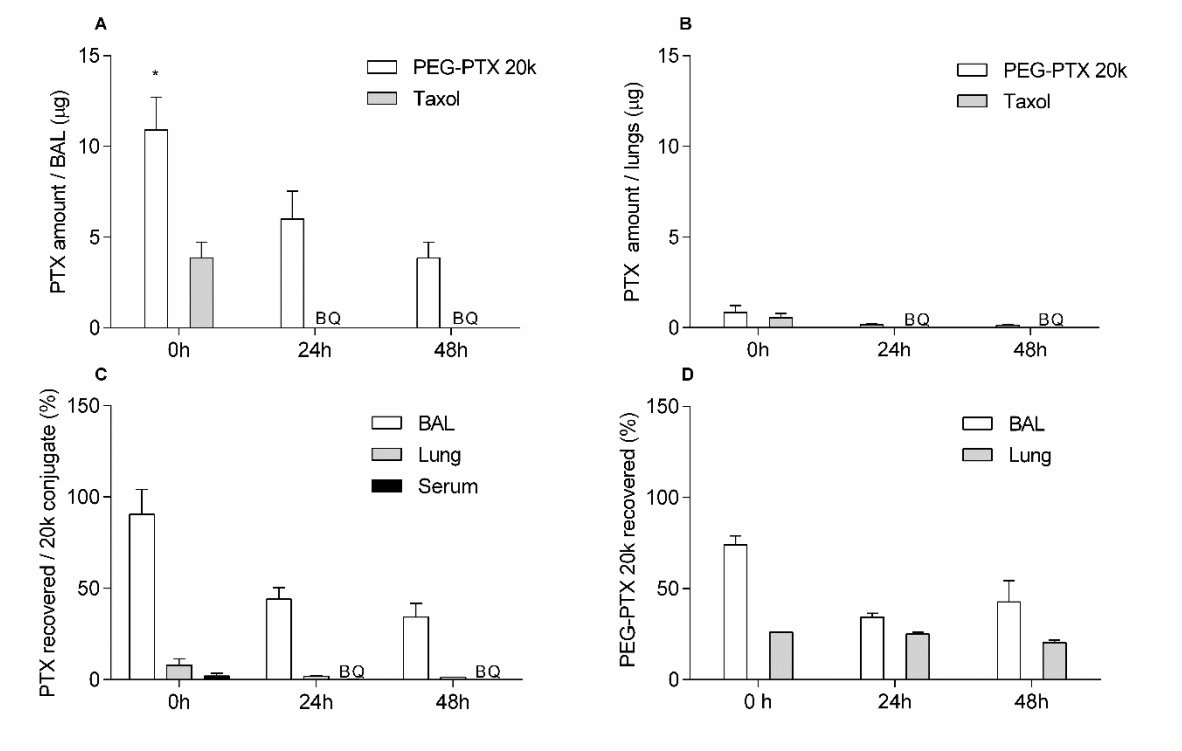

Fig. 8 PTX and PEG-PTX 20k recovered from the respiratory tract 0h, 24h and 48h post intratracheal administration. (A) PTX amount in BAL and (B) PTX amount in the lungs of mice having received PEG-PTX 20k (20 mg/kg PTX equiv., 400 µg PTX equiv. per mouse) and Taxol® (0.5 mg/kg, 10 µg per mouse). (C) Amount of PTX recovered from BAL, lungs and serum post intratracheal administration of PEG-PTX 20k, expressed as a percentage of the total dose recovered at time 0. (D) Amount of PEG-PTX 20k recovered from BAL and lungs expressed as a percentage of the total dose recovered at time 0. * p <0.05, when compared with Taxol® group (Mann-Whitney); BQ, below quantification limit.

4. Discussion

The present study demonstrated that PEG-PTX conjugates had lower toxicity but superior anti-tumor efficacy than Taxol® when administered i.t. to mice. We used PEGylation to solubilize PTX instead of Cremophor EL, which reduced the local toxicity. Moreover, the choice of large MW of PEG (20 kDa) prolonged the retention time of paclitaxel in the lungs, which led to the improved efficacy. These results suggest that PEGylation of chemotherapeutics could be an effective approach to embody inhaled chemotherapy in the future.

Conjugation of PTX to PEG largely increased the MTD following intratracheal instillation. The MTD of PEG-PTX 6k i.t. was 100-fold of Taxol® i.t.. This could be attributed to the prodrug

nature of PEG-PTX conjugates. Intratracheal delivery of PEG-PTX 6k at a high dose (50 mg/kg PTX equiv.) increased LDH and neutrophils levels in BAL 24h post-delivery. However, delivery of equiv. dose of PEG-N_3 6k did not increase LDH and neutrophils significantly. Therefore, the toxicity of conjugates mainly came from the high dose of PTX itself. Taxol® containing 50% (v/v) of Cremophor EL was used as the PTX control in this study. Intratracheal delivery of either Taxol® or Cremophor EL alone caused increased levels of total proteins, LDH and neutrophils, which indicated that the pulmonary toxicity was induced by both PTX and the Cremophor EL. Although it is not suitable for pulmonary delivery, Cremophor EL is needed in the formulation to solubilize the desired amount of PTX. Further, PEG-PTX 6k delivered at the same low dose as Taxol® (0.5 mg/kg PTX equiv.) did not induce significant toxicity to the lungs. These results suggest that PEGylation reduced the local toxicity of the native drug and that the local toxicity of PEG-PTX conjugates was dose-dependent.

As PEG-PTX 20k presented long retention time in the lungs, toxicological concerns may arise from long-term accumulation of high molecular weight PEG in the lungs. The safety of PEG 3,350 Da has been proven in rats following 2-week exposure by aerosol [29]. However, there is no safety study on large PEG (> 5 kDa) delivered by inhalation. In this study, PEG-N_3 6k at 200 mg/kg (50 mg/kg PTX equiv.; 5 mg of PEG-N_3 6k per mouse) presented acceptable safety over 7 days following a single administration dose in the lungs. Generally, PEG is non-toxic and extensively used in drug delivery. However, the non-biodegradable property of PEG might be the drawback for application by inhalation. It is anticipated that either PEG-PTX or the free PEG released following the hydrolysis of PEG-PTX would be partially cleared by mucociliary clearance and by alveolar macrophages. The fraction absorbed in the systemic circulation would likely be eliminated by renal clearance [30]. Considering the large PEG size and the high

doses delivered, long-term safety studies are needed to ascertain the safety of inhaled PEG-PTX.

43% and 20% of the PEG-PTX 20k initial dose were still present in BAL and lungs 48h post-intratracheal instillation, respectively. In previous studies of PEG retention in the lungs, PEG with MW > 5 kDa was not quickly cleared and absorbed into the systemic circulation [20]. In addition, 60% of the initial dose of PEG 40 kDa remained in murine lungs 48h post-intratracheal instillation [31]. Therefore, the prolonged retention of PEG-PTX 20k was probably due to the large MW of PEG.

As compared with other drug delivery systems, PEG-PTX showed increased retention time of the drug in the lungs. Koshkina et al. investigated the delivery of liposomal PTX by aerosol inhalation [11]. PTX was released and reached the concentration peak in the lungs after 30 min of inhalation but it was cleared within 3 hours. Gill et al. studied the administration of PTX micelles by the pulmonary route [12]. This system showed prolonged drug retention of PTX in the lungs compared to Taxol® administrated intratracheally. Paclitaxel concentration at 12h post-delivery was found to be 46% of that at 1h post-delivery. Kaminskas et al. assessed the pulmonary delivery of a PEGylated polylysine dendrimer of doxorubicin [14]. They demonstrated that 20% of the initial dose of the doxorubicin dendrimer was present in the BAL and the lungs 1 day after the intratracheal instillation.

The long retention of PEG-PTX conjugates in the lungs is attributed to a slow pulmonary absorption due to the increased MW compared with free PTX as well as to the mucoadhesion of large PEG molecules [31]. Nevertheless, the formation of conjugate nanostructures such as micelles or aggregates could also contribute to the increased residence time of the conjugates in the lungs by decreasing diffusivity and by entrapping PTX. We analyzed the PEG-PTX 6k

solution by dynamic light scattering (DLS) method. No aggregate was observed at the concentration of 0.5 mg/mL. However, aggregates at around 100 nm and 1 μm were found at a concentration of 40 mg/mL. The polydispersity was too high to calculate an accurate size distribution. The aggregates at 100 nm could be micelles. However, the DLS data were not convincible to ascertain the formation of micelles. PEG itself forms aggregates in solution. In a previous study in our group, aggregates around 100 nm were visible by DLS in a solution of PEG 40k only. Therefore, conjugates are more likely to randomly aggregate at very high concentration due to the higher chance of conjugate molecules to encounter and bind.

It is anticipated that the slightly acidic microenvironment of the tumor would lead to accelerated cleavage of PTX from the conjugate backbone compared with the non-tumor area [32, 33]. Therefore, the hydrolysis of PEG-PTX could possibly take place prior to the internalization of the conjugates, which possibly resulted in a high proportion of PTX in the interstitial tumor area within the lungs. It is likely that the PTX concentrations achieved using PEG-PTX was high enough to improve lung tumor exposure to PTX, because PEG-PTX showed significantly increased tumor regression compared with the intravenously or intratracheally injected Taxol®.

The intratracheal delivery of PEG-PTX 6k and 20k conjugates demonstrated superior anti-tumor efficacy than both i.v. and i.t. administered Taxol®, the formulation of the free drug. This result suggests that both the local delivery and the PEGylation of PTX contributed to the enhanced efficacy. As discussed above, PEG-PTX also demonstrated lower local toxicity than Taxol®. Therefore, our hypothesis that PEGylation can improve the anti-tumor efficacy with reduced local toxicity is demonstrated. Moreover, since PEG-PTX 20k at a lower dose than

PEG-PTX 6k showed equivalent anti-tumor efficacy but less toxicity, it could be assumed that better efficacy and lower toxicity could be achieved with higher molecular weight of PEG.

As a summary, PEGylated PTX can increase anti-tumor efficacy compared to Taxol® post i.t. delivery. PEGylated paclitaxel can also greatly improve the MTD i.t. and decrease local toxicity. The retention time of PTX can be prolonged by conjugation to PEG with large MW. Therefore, PEG-PTX conjugates are a promising system for application in inhaled chemotherapy.

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