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Co-administration of tLyp-1 with polymeric paclitaxel conjugates: Enhanced intratumoral accumulation and anti-tumor efficacy

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Background: It has been previously demonstrated that conjugation of paclitaxel to a linear poly(l-γ-glutamylglutamine) backbone can enhance water solubility of paclitaxel. However, intratumoral penetration of the nanoscale poly(l-γ-glutamylglutamine)-paclitaxel conjugate (PGG-PTX) was still limited due to dysfunctional tumor blood vessels as well as high interstitial pressure in the tumor microenvironment. *Purpose*: The objective of the present research was to investigate the feasibility of co-administration of a tumor penetration enhancing peptide tLyp-1 for improving intratumoral accumulation and consequent anti-tumor efficacy of PGG-PTX. *Methods*: The influence of co-administration of tLyP-1 with PGG-PTX on intratumoral accumulation (*via* HPLC-MS/MS) and anti-tumor efficacy (by monitoring the change in the tumor volume) was investigated using a breast cancer (4T1) tumor-bearing mouse model. In addition, the systemic toxicity of co-administration of tLyP-1 with PGG-PTX was assessed by monitoring the change in the animal body weight. *Results*: It was observed that co-administration of tLyP-1 with PGG-PTX dramatically improved PGG-PTX accumulation in the tumors, resulting in improved inhibition efficiency against tumor growth. Moreover, co-administration of tLyP-1 with PGG-PTX did not change the systemic toxicity profile of PGG-PTX. *Conclusion*: Co-administration of tLyp-1 may be a promising strategy for improving the passive tumor-targeting performance of polymeric drug conjugates.

1. Introduction

Polymer-drug conjugates have attracted increasing attention for cancer therapy, owing to their advantages such as improved solubility of chemotherapeutics, unique drug release characteristics, excellent serum stability, as well as reduced drug resistance (Delplace et al. 2014). Over the past few years, both preclinical studies and clinical trials on polymer-drug conjugates for anticancer therapy have grown exponentially (Pérez-Herrero and Fernández-Medarde 2015).

Paclitaxel (PTX), a microtubule-stabilizing agent, has been successfully used for the treatment of various cancers. Due to its extremely poor water solubility, organic solvents and/or surfactants (*e.g.* a mixture of ethanol and cremophor (polyethoxyated castor oil)) are needed in order to solubilize PTX for intravenous administration purposes. However, cremophor is often associated with severe toxicities such as neurotoxicity, nephrotoxicity, vasodilatation lethargy, as well as hypersensitivity reactions that are non-negligible, requiring premedication with corticosteroids and antihistamines (Wiernik et al. 1987; Weiss et al. 1990; Rowinsky et al. 1993).

Chemical conjugation of PTX to water-soluble biodegradable polymers such as polyethylene glycol (PEG) (Liang et al. 2012), poly (ethylene glycol)-b-poly (L-lysine) (Lv et al. 2014), and poly (γ-glutamic acid) (γ-PGA) (Seth et al. 2014) can improve water solubility of PTX and hence avoid the use of unsafe surfactants. In our previous research, a biodegradable linear polymer, poly (L-γ-glutamylglutamine) (PGG), was used as the polymeric vehicle (structure shown in Fig. 1) for covalent conjugation of PTX. The PGG-PTX conjugate not only possessed much higher water solubility (100 mg/ml) compared to PTX (0.006 mg/ml), but also demonstrated good therapeutic and safety performance in several animal models investigated (Feng et al. 2010; Van et al. 2010; Yang et al. 2012).

The PGG-PTX conjugate exists in aqueous environment as nanoparticles (~20 nm) (Chuan et al. 2014) and so it is not clear why this would

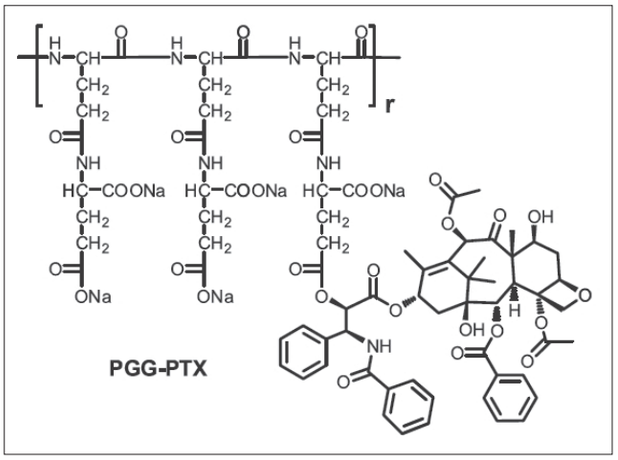

Fig. 1: Chemical structure of the PGG-PTX conjugate.

affect/hamper intratumoral penetration. Intratumoral penetration may be limited by the unfriendly tumor microenvironment including dysfunctional blood vessels and high interstitial pressure (Khawar et al. 2015). Tumor penetration enhancing peptides, which usually contain the sequence motif R/KXXR/K (also termed as the C-end Rule motif, or CendR), has previously been used to improve tumor penetration and thereby anticancer efficacy of chemotherapeutics (Sugahara et al. 2010). The activity of tumor penetration enhancing peptides mainly relies on their binding to neuropilin-1 (NRP-1), which is a transmembrane protein overexpressed on the endothelial cells of tumor blood vessels (Chaudhary et al. 2014; Prud'homme and Glinka 2012). For example, tLyP-1 (CGNKRTR, possessing CendR modif) constructed by Ruoslahtis's group (Roth et al. 2012) has been reported to improve tumor penetration of both small molecules and nanoscale drug carriers *via* a NRP1-dependent internalization pathway. tLyP-1 can either be covalently conjugated (Choi et al. 2013; Hu et al. 2013a; Liu et al. 2015; Xu et al. 2014; Yang et al. 2014a; Yang et al. 2014b), or simply co-administered with small molecules or nanocarriers (Hu et al. 2013b; Miao et al. 2014). However, until now whether tLyP-1 is capable of enhancing tumor accumulation of polymeric drug conjugates has not been studied. In the present research, the influence of co-administration of tLyP-1 with PGG-PTX conjugates on both intratumoral accumulation and tumor growth inhibition efficacy was investigated using a 4T1 tumor-bearing mouse model.

2. Investigations and results

2.1. Enhanced intratumoral accumulation

A HPLC-MS/MS methodology has been established and validated with respect to accuracy, precision, specificity, matrix effect as well as sample stability for quantification of PGG-PTX in tumor tissue (Figs. 2a and 2b). As shown in Figure 2c, co-administration of tLyp-1 significantly increased the distribution of PGG-PTX in tumor tissues.

2.2. Enhanced tumor growth inhibition effect

Figures 3a and 3b show that the co-administration of tLyP-1 together with PGG-PTX led to slower tumor growth compared to PGG-PTX alone. tLyp-1 itself did not inhibit tumor growth. In addition, the body weight profiles of mice treated with PGG-PTX conjugates plus tLyp-1 were similar to those with PGG-PTX alone (Fig. 3c), preliminarily proving the safety of the co-administration of tLyp-1 with PGG-PTX.

3. Discussion

There is substantial physical resistance to drug delivery in the tumor microenvironment, which was associated with pathophysiological components including abnormal and poorly perfused vasculature, elevated interstitial fluid pressure, growth-induced solid stress and solid stress from abnormal stromal matrix. Nowadays different approaches have been utilized to overcome these transport barriers in the tumor microenvironment, including vascular normalization, solid stress alleviation and tumor penetration enhancing peptides. Co-administration of tumor-penetration enhancing peptides with nanocarriers resulted in enhanced intratumoral accumulation of chemotherapeutics. For example, the intratumoral accumulation of paclitaxel-albumin nanoparticles (Abraxane®) was improved by co-administration of iRGD of the nanoparticles (Sugahara et al. 2010). Although it has been demonstrated that enhanced tumor penetration of nanocarriers can be achieved by simple co-administration of tumor penetration enhancing peptides with the nanocarriers, whether the same strategy can be utilized to improve tumor accumulation of polymer-chemotherapeutic conjugates was still unknown. In the present study, the possibility of enhancing the intratumoral delivery of polymer-chemotherapeutic conjugates *via* simple co-administration of tumor penetration enhancing peptides was investigated. The PGG-PTX conjugate with excellent water solubility (Yang et al. 2012) was chosen as a model polymer-chemotherapeutic conjugate, and tLyp-1 was selected as a model tumor penetration enhancing peptide. Co-administration of tLyp-1 significantly enhanced the intratumoral concentration of the PGG-PTX conjugate, resulting in slower tumor growth in comparison with the PGG-PTX conjugates alone since more polymeric conjugates had been delivered into the tumors. More polymeric conjugates inside the tumor allowed more paclitaxel to reach and kill cancer cells, thus improving tumor growth inhibition efficacy. It is not clear why this would affect/hamper intratumoral penetration. This was in accordance with literature on iRGD that enhanced intratumoral accumulation of Abraxane® was accompanied by significantly improved anti-tumor efficacy (Sugahara et al. 2010) and prolonged survival time of B16F1-bearing mice when co-administered together with cisplatin-loaded poly(l-glutamic acid)-*g*-methoxy poly(ethylene glycol) nanoparticles (Yu et al. 2015). Only tLyp-1 did not influence the growth of tumors, indicating tLyP-1 itself is safe and not cytotoxic.

Co-administration of tLyP-1 did not influence the systemic toxicity of the PGG-PTX conjugates (Fig. 3b). As proven by Ruoslahti et al, tLyP-1 peptide showed robust and selective homing capability to tumors. This peptide can penetrate from the blood vessels into the tumor parenchyma and improve extravasation of a co-injected nanoparticle into the tumor tissue (Roth et al. 2012). An important feature of CendR peptides including tLyP-1 is its ability to deliver systemically co-injected bystander free molecules or nanoparticles into tumor tissue, using the bulk intratumoral transport mechanism which is activated through the vasculature and also within the tumor tissue. The binding affinity and activation capability of tLyP-1 with neurophilin-1 (NRP-1) and neurophilin-2 (NRP-2) has been suggested as the basis of the bulk intratumoral transport mechanism triggered by co-injected tLyP-1 (Ruoslahti 2012). The tumor cell line (4T1) used in this study has been proven to overexpress both NRP1 and NRP-2 by Ruoslahti et al. (Ruoslahti 2012). Ruoslahti's research demonstrated the ability of tLyP-1 to trigger tumor penetration of co-injected iron oxide nanoparticles dubbed nano-worms by activating the CendR pathway in 4T1 tumor-bearing mice. Another

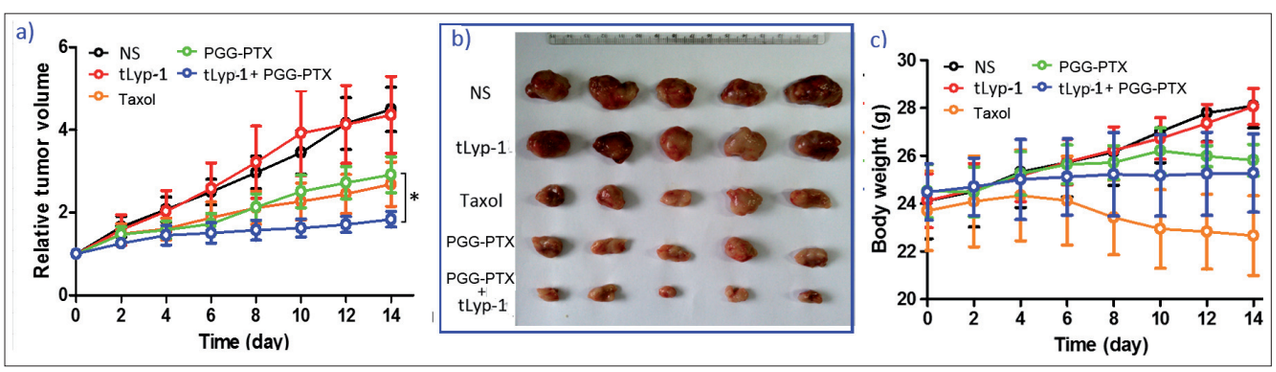

Fig. 2: HPLC-MS/MS analysis of PGG-PTX concentration in 4T1 tumor samples. a) Standard curve of peak ratio (analyte: internal standard) vs. PGG-PTX concentration in tumor homogenate. b) Representative chromatograph of tumor samples. c) Comparison of the intratumoral concentration of PGG-PTX conjugates vs. PGG-PTX + tLyp-1 (mean ±S.D., *n*=5)

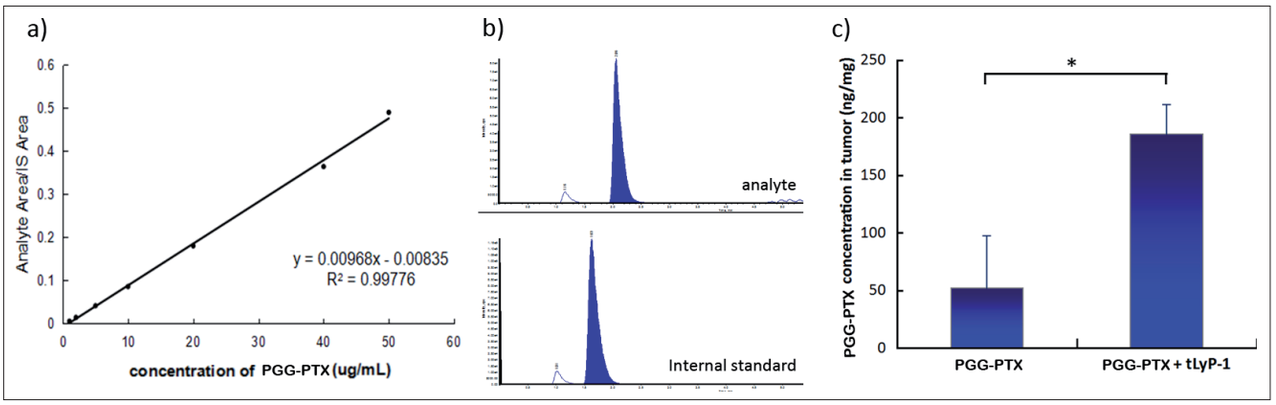

Fig. 3: The tumor growth inhibition and safety profiles of PGG-PTX conjugates in 4T1 tumor-bearing mice. a) Change in relative tumor volumes compared with the initial tumor size at day 0 (mean ±S.D., n=5). b) Photographs of tumors excised after sacrifice of animals at day 14. c) Change of body weight of animals (mean ±S.D., n=5)

literature report (Miao et al. 2014) confirmed that co-injection of tLyP-1 can enhance the intratumoral penetration and accumulation of lactoferrin-modified nanoparticles in glioma-bearing mice. Therefore, we hypothesized that the enhancement of PGG-PTX intratumoral accumulation by tLyP-1 might also be mediated by the bulk intratumoral transport mechanism. Further studies will be conducted to elucidate the mechanism(s) of how tLyP-1 improves the intratumoral penetration of polymeric drug conjugates.

Our investigation indicated that a simple co-administration of the tumor penetration peptide tLyp-1 significantly enhanced intratumoral accumulation and thereby improved tumor growth inhibition efficacy of PGG-PTX conjugates. This "proof-of-concept" study was very encouraging and informative for future application of tumor penetration enhancing peptides for improving intratumoral delivery of polymeric prodrugs.

4. Experimental

4.1. Materials

PGG-PTX conjugates were synthesized based on previously reported methods (PTX content: ~ 35%) (Van et al. 2010). Taxol® was purchased from BMS Co. Ltd (United States). tLyP-1 (CGNKRTR) was synthesized by Dalian Melun Biology Technology Co. Ltd. Acetonitrile (HPLC grade), formic acid (HPLC grade), ammonium formate (HPLC grade), RPMI 1640 cell culture media and fetal bovine serum were purchased from Merck (Germany). Cephalomannine (HPLC grade) was purchased from J&K Chemical Co. Ltd (Shanghai, China). 4T1 cells were purchased from ATCC Co. Ltd (United States). Other chemicals were purchased from Meilun Biology Technology Co. Ltd (Dalian, China).

4.2. Animals

Male BALB/c mice were purchased from SLRC Laboratory Animal Co. Ltd (Shanghai, China) and housed under pathogen-free conditions in the Experimental Animal Center of the School of Pharmacy, Fudan University. All animal experimental procedures were approved by the Ethics Committee of the School of Pharmacy, Fudan University.

4.3. Establishment of a tumor-bearing mouse model

Mouse breast cancer 4T1 cells were cultured in the RPMI-1640 medium containing 10% (v/v) fetal bovine serum and 1% penicillin-streptomycin at 37 °C in a 5% CO_2 humidified incubator. 4T1 cells (2×10^5, re-suspended in 5 µl sterile phosphate buffer per mouse) were subcutaneously inoculated near the right armpit of BALB/c mice to establish the tumor-bearing mouse model.

4.4. Establishment and validation of a bioanalytical method

4.4.1 HPLC-MS/MS condition

HPLC-MS/MS analysis was conducted on Agilent 1200 HPLC coupled with Applied Biosystems/MDS Sciex API 4000 Q TRAP equipped with Phenomenex Synergi Polar-RP column (50×2 mm). The mobile phase was a mixture of water:acetonitrile (pH adjusted to 2.05 by 5 mM formic acid-ammonium formate buffer) using appropriate gradient. Cephalomannine was used as the internal standard (IS).

4.4.2. Sample treatment

For standard curve preparation or quality control (QC), 50 µl of blank tumor homogenate and 50 µl of a series of PGG-PTX aqueous solutions in water were well mixed. For tumor samples excised from mice, 50 µl of tumor homogenate were mixed with 50 µl of pure water. The following procedures were conducted: *1)* washing with acetonitrile three times (in order to remove free PTX); *2)* addition of the working internal standard (10 µg/ml cephalomannine) to all samples (except for the double blank); *3)* addition of 0.5 ml 3M HCl to hydrolyze PGG-PTX at 85 °C for 0.5 h; *4)* neutralization with 5 M sodium acetate; *5)* extraction PTX from tumor tissues with ethyl acetate; *6)* drying the supernatant under nitrogen at 40 °C; and *7)* reconstitution the dry film with the mobile phase.

4.5. In vivo tumor growth inhibition experiment

Five days post-tumor cell inoculation, mice were randomly assigned into 5 groups (5 animals each group) and treated with: (1) Group 1: normal saline; (2) Group: Taxol® (dose: 10 mg/kg); (3) Group 3: tLyP-1 (dose: 5 mg/kg); (4) Group 4: PGG-PTX (dose: 30 mg/kg); and (5) Group 5: PGG-PTX (dose: 30 mg/kg) plus tLyP-1 (dose: 5 mg/kg) *via* the tail vein at days 0, 3, 6, and 9. The weight of the animals, the weight and size of the tumors were monitored. The volume of the tumor was calculated using the following equation: tumor volume (mm^3) = (length × $width^2$)/2, where the length and width are in mm. The tumor size of each mouse at different time points was expressed as % relative to the initial tumor size of the corresponding animal at the beginning of treatment.

4.6. Distribution of PGG-PTX in tumor

When the tumor volume reached 50 mm^3, the PGG-PTX conjugate (30 mg/kg) was intravenously administered alone or together with tLyP-1 (dose: 5 mg/kg) into the tumor-bearing mice. Three hours following the administration, the mice were anesthetized with 5% chloral hydrate and exsanguinated. Tumor samples were excised and analyzed for PGG-PTX concentrations using HPLC-MS/MS as described in 5.4.

4.7. Statistics

Difference between groups were analyzed using Student's t-test, and differences were considered to be significant at a level of $p < 0.05$.

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Conflicts of interest: None declared.

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