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Research Paper

Renal targeted delivery of triptolide by conjugation to the fragment peptide of human serum albumin

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ABSTRACT

We have previously demonstrated that peptide fragments (PFs) of the human serum albumin could be developed as potential renal targeting carriers, in particular, the peptide fragment, PF-$A_{299–585}$ ($A_{299–585}$ representing the amino acid sequence of the human serum albumin). In this paper, we conjugated triptolide (TP), the anti-inflammatory Chinese traditional medicine, to PF-$A_{299–585}$ via a succinic acid spacer to give TPS-PF-$A_{299–585}$ (TP loading 2.2% w/w). Compared with the free TP, TPS-PF-$A_{299–585}$ exhibited comparable anti-inflammatory activity in the lipopolysaccharide stimulated MDCK cells, but was significantly less cytotoxic than the free drug. Accumulation of TPS-PF-$A_{299–585}$ in the MDCK cells *in vitro* and in rodent kidneys *in vivo* was demonstrated using FITC-labeled TPS-PF-$A_{299–585}$. Renal targeting was confirmed *in vivo* in a membranous nephropathic (MN) rodent model, where optical imaging and analyses of biochemical markers were combined to show that TPS-PF-$A_{299–585}$ was capable of alleviating the characteristic symptoms of MN. The collective data affirm PF-$A_{299–585}$ to be a useful carrier for targeting TP to the kidney.

1. Introduction

The kidneys are vital organs of humoral regulation and their dysfunction leads to an imbalance of nutrient and metabolite levels in the body. Chronic kidney disease (CKD), a serious public health issue in many countries [1,2], requires expensive pharmacotherapeutic interventions and, eventually, dialysis or renal transplantation to sustain the lives of patients. Drugs for the treatment of renal diseases often lack specificity of action, and are associated with complicated extra-renal toxicity. These drugs may benefit from selective targeting to kidneys to improve their efficacy and toxicity profiles [3–5].

Triptolide (TP) is a novel bioactive diterpenoid epoxide isolated from *Tripterygium wilfordii Hook F.*, an established Chinese traditional medicine that has promising potential to treat a variety of renal diseases [6]. TP exhibits potent immunosuppressive and anti-inflammatory properties [5,7], but its progression into clinical use is hampered by poor renal discriminative activity, which results in severe side effects on the circulatory and reproductive systems [8]. Consequently, much research has been focused on attenuating the toxicity of TP by targeting its delivery to the kidney using pharmaceutical strategies [9–11].

Peptide fragments (PFs) of the human serum albumin have previously been shown by our laboratory to accumulate in the kidney after parenteral administration, which may allow them to be developed into renal targeting ligands [12]. Among the peptide fragments examined, PF-$A_{299–585}$ showed the greatest promise, with about 40% of the injected doses accumulating in the kidneys at 1 h post-injection, presumably via the endocytic pathway mediated by the cubilin/megalin receptor on the apical membrane of proximal tubular cells that is known to transport low molecular weight proteins [13]. The aim of this study was to clarify the renal targeting potential of PF-$A_{299–585}$ as a drug carrier. To this end, PF-$A_{299–585}$ was chemically conjugated to TP via a succinic acid spacer, and the resultant product, 14-succinate triptolide-fragment peptide (TPS-PF-$A_{299–585}$), was evaluated for its cellular uptake and anti-inflammatory activities using an *in vitro* lipopolysaccharide stimulated MDCK cell model and an *in vivo* membranous nephropathy rodent model. The collective data affirm the capability of TPS-PF-$A_{299–585}$ to accumulate in renal cells and to alleviate biochemical markers associated with renal inflammation.

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2. Materials and methods

2.1. Materials and animals

TP was purchased from Xieli Biotechnology Co. Ltd. (Sichuan, China). Succinic acid, fluorescein isothiocyanate (FITC) and bovine serum albumin (BSA) were obtained from Sigma–Aldrich Co. Ltd. (Gillingham, UK). PF-$A_{299-585}$ was prepared according to methods reported in our previous paper [12]. MDCK cells were from the American Type Culture Collection (VA, USA). Hyclone fetal bovine serum and Dulbecco's modified Eagle's medium (DMEM) were supplied by Thermo Scientific Co. Ltd. (IL, USA). Freund's complete adjuvant was from Difco (MI, USA). All other chemicals and solvents were of the analytical grade.

Kunming mice (20 ± 2 g, male) and Sprague Dawley rats (200 ± 20 g, male) were obtained from the Da-Shuo Experimental Animal Ltd. (Beijing, China). The animals were allowed to acclimatize for a few days in environmentally controlled quarters (24 ± 1 °C, 12 h light/dark cycle) and, unless specified otherwise, were provided with water and food ad libitum. All animal studies were approved by the Sichuan Academy of Chinese Medicine Sciences Animal Ethical Experimentation Committee (SYXK (Chuan)2013-100), and were performed according to the requirements of the People's Republic of China National Act on the use of experimental animals.

2.2. Synthesis of TPS-PF-$A_{299-585}$ and FITC-labeled TPS-PF-$A_{299-585}$

The synthesis of TPS-PF-$A_{299-585}$ and FITC-labeled TPS-PF-$A_{299-585}$ was carried out according to previous reports [5,14]. Triptolide succinate (TPS) was first synthesized by dissolving 100 mg TP with an excess of succinic anhydride (150 mg) (molar ratio of TP:succinic anhydride ~ 1:5), together with 10 mg of 4-dimethylaminopyridine in 3 ml of anhydrous CH_2Cl_2, and stirring the reaction mix overnight at ambient temperature after the addition of 2 ml trimethylamine. The product was washed thoroughly with distilled water and purified by isocratic elution through a silica column using chloroform:methanol (20:1, v/v) as mobile phase. TPS was identified using ^{1}H NMR (Bruker Avance III 400 MHz spectrometer) and MS (Bruker amaZon SL, Bremen, Germany) analyses. TPS-PF-$A_{299-585}$ was synthesized by adding dropwise a solution comprising 100 mg TPS (~210 μmol), 100 mg 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride and 50 mg N-hydroxybenzotriazole dissolved in 2 ml acetonitrile, to a PF-$A_{299-585}$ solution comprising 100 mg PF-$A_{299-585}$ (~3 μmol) dissolved in 20 ml of 0.1 M borate buffer. The reaction system was stirred at 4 °C overnight and the product was purified by dialysis against distilled water. FITC labeling was provided by reacting 20 ml of TPS-PF-$A_{299-585}$ solution (1 mg/ml in pH 7.2 phosphate buffered saline (PBS), ~0.6 μmol) with 1 ml of FITC solution (1 mg/ml in dimethylsulfoxide (DMSO), 2.6 μmol) for 2 h in the dark, and purifying the product by dialysis against distilled water. The product was purified by dialysis against distilled water. All products were freeze dried for storage at −20 °C prior to use.

TP was quantified using an ACQUITY UPLC (Waters, MA, USA) system equipped with a tunable UV detector and an autosampler. TP was eluted at a flow rate of 0.6 ml/min in a BEH C_{18} column (50 × 2.1 mm, 1.7 μm) at 30 °C using an isocratic mobile phase of methanol:water (40:60 v/v) and detection wavelength of 218 nm. The injection volume was 5 μl and the total run time for each sample was 5 min. The regressive equation of peak area versus concentration for TP standard solutions in acetonitrile (2.16–108.0 μg/ml) was $y = 18450x + 8056$ ($r^2 = 0.9999$).

TP conjugation efficiency was determined after the hydrolysis of the conjugate with NaOH [5]. A 0.5 ml aliquot of TPS-PF-$A_{299-585}$ (2 mg/ml in distilled water) was mixed with 25 μl of 1 M NaOH at 25 °C, and after 10 min, the mixture was neutralized with 25 μl of 1 M HCl, followed by the addition of 0.45 ml of mobile phase. After vortexing, 5 μl of the supernatant was injected into the UPLC system to measure the TP level.

FITC labeling efficiency was determined fluorospectrophotometrically (λ_{ex} = 492 nm, λ_{em} = 519 nm) with reference to FITC using a previous method established in our laboratory [15], and FITC standard solutions in the concentration range of 0.5–16.0 ng/ml in carbonate buffer (pH = 9.0) (F-4600 fluorospectrophotometer, Hitachi, Japan). A linear regressive equation of $y = 60.275x - 5.979$, with r^2 value of 0.9996, was established. A weighed amount of FITC-labeled TPS-PF-$A_{299-585}$ was then dissolved in carbonate buffer, and its fluorescence level was translated to FITC labeling efficiency using the regressive equation.

2.3. In vitro release of TP from TPS-PF-$A_{299-585}$

In vitro release of TP from the TPS-PF-$A_{299-585}$ conjugate was evaluated using phosphate buffer solutions having the pH range of 3.0–8.0. Samples were incubated at 37 ± 0.5 °C for a total period of 6 h. At predetermined intervals, 0.5 ml aliquots were withdrawn from each sample, thoroughly mixed with 1 ml acetonitrile, and the TP content of the aliquot was determined using the UPLC method.

The *in vitro* release of TP from the TPS-PF-$A_{299-585}$ conjugate was also evaluated using plasma and kidney homogenate obtained from euthanized adult SD rat as dissolution media. Plasma was separated from the rodent whole blood by centrifugation with 1% heparin for 10 min at 4 °C at 2000 rpm (3K30 centrifuge, Sigma, Germany), and diluted with an equal volume of normal saline. Kidney homogenate was prepared by homogenizing the kidneys with 2 times its volume of saline. TPS-PF-$A_{299-585}$ was dispersed in 10 ml of plasma or kidney homogenate to a final concentration of 0.5 mg/ml, and the samples were incubated at 37 ± 0.5 °C for 12 h. At 5 min, 10 min, 15 min, 30 min 1 h, 2 h, 6 h, and 12 h, 0.5 ml aliquots were withdrawn and thoroughly mixed with 1 ml acetonitrile. After centrifugation at 15000 rpm for 5 min at 4 °C, TP concentration in the supernatant was determined using the established UPLC method. Regressive equations obtained for standard solutions of plasma and kidney homogenates spiked with TP in the concentration range of 2.16–43.2 μg/ml were $y = 5866x + 4483$ ($r^2 = 0.9990$) and $y = 4525x + 2368$ ($r^2 = 0.9980$), respectively.

2.4. In vitro cellular assays

2.4.1. Cytotoxicity

MDCK cells (passage 15) were seeded onto 96-well plates (Corning, NY) at a density of 1×10^4 cells/well with 200 μl of DMEM. After incubation for 24 h in a CO_2 incubator (Thermo Scientific, Marietta, OH), the culture medium was replaced with 200 μl of TP (0.11–22.00 μg/ml in DMEM with 1% DMSO) or TPS-PF-$A_{299-585}$ (at equivalent TP concentration of 0.11–22.00 μg/ml in DMEM) solutions, and the plates were returned to the incubator for another 24 h. The test samples were discarded and the viability of cells was determined by adding 20 μl of MTT (5 mg/ml in PBS) and 100 μl DMEM into each well. The medium was aspirated after 4 h of incubation, and the intracellular formazan solubilized with 150 μl DMSO was quantified by measuring the optical density of each well at 490 nm (iMark microplate-reader, Bio-rad, CA, USA).

2.4.2. In vitro anti-inflammatory activity

The *in vitro* anti-inflammatory activity of TPS-PF-$A_{299-585}$ was measured using lipopolysaccharide (LPS)-stimulated MDCK cells