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A renal-targeted triptolide aminoglycoside (TPAG) conjugate for lowering systemic toxicities of triptolide

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ABSTRACT

Triptolide (TP), a naturally derived compound, is proven effective in the treatment of nephritis and chronic allograft nephropathy. However, the severe multiorgan toxicity greatly limited it from further clinic use. 2-Glucosamine was demonstrated as a potential targeting ligand that could specifically interact with megalin receptors highly expressed in renal proximal tubules. In this study, 2-glucosamine was employed as a glycosyl donor while triptolide the acceptor to afford a nonhydrolyzable triptolide derivative-triptolide aminoglycoside (TPAG). The kidney-targeting efficiency, pharmacodynamic properties and safety of TPAG were thus evaluated. TPAG displayed 6.94-fold of $AUC_{0-t,\,kidney}$ and 13.96-fold of $MRT_{0-t,\,kidney}$ compared to TP. Additionally, TPAG presented improved protective effect against renal ischemia/reperfusion injury. Compared to TP's multiorgan toxicity, TPAG showed minimum toxicity toward the kidney and genital systems, and greatly lowered toxicity in the liver and immune systems. In sum, our study presented an alternative structure modification of triptolide with improved safety and efficacy profiles.

1. Introduction

Kidney failure is a public health problem with increasing prevalence worldwide [1]. In the United States, patients with end-stage renal disease (ESRD) represent about 0.12% of the total US population. The prevalence of ESRD is even higher in Japan at 0.16% [2]. For ESRD patients, renal transplantation and dialysis have remained the major treatment options [3]. With improved early allograft survival, chronic allograft nephropathy has become the dominant cause of kidney-transplant failure [4]. Current immunosuppressive drugs have dramatically improved the management of acute graft rejection but fail to control chronic rejection [5]. Thus, developing improved immunosuppressive regimens will be of great significance.

Triptolide (TP), a diterpenoid triepoxide isolated from a Chinese herbal plant (*Tripterygium Wilfordii* Hook F) has been shown effective in the treatment of nephritis and chronic allograft nephropathy [6]. However, its clinical use was greatly restricted by multiorgan toxicity and poor water solubility (The logP of triptolide in octanol/water system was 1.494). TP induces oxidative stresses which contribute to acute nephrotoxicity [7]. TP was also proven to induce testicular toxicity by inhibiting proliferation of spermatogenic cells at low concentrations [8]. Liver toxicity of TP has been reported as observed by lipid peroxidation in hepatocytes [9]. The immunotoxicity was caused by drug-induced apoptotic death of T lymphocyte [10]. To address these limitations that hinder the druggability, we proposed a novel conjugate by rational structural modification utilizing 2-glucosamine as the targeting ligand which aimed to improve the solubility, targeting efficiency and lower the systemic toxicity of TP *in vivo*.

Lin et al. [11] has found that a novel moiety 2-glucosamine would significantly improve the uptake of prednisolone in the kidneys and it was proven a potential ligand of megalin receptors. Megalin receptor, with molecular weight of 600 kD, is a protein expressed in the renal tubule epithelium [12]. 2-Glucosamine was identified as a ligand of megalin and it

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mediates kidney-targeted drug delivery [11]. Encouraged by these achievements, Zhou et al. [13] made further progress by conjugating glucosamine to triptolide (TPG) *via* a readily hydrolyzable carbamate bond to improve the uptake of triptolide in the kidneys. However, TPG has several limitations: carbamate linkage was biologically unstable and was subjected to rapid metabolization by certain cholinesterases, aliesterases and plasma albumin. TPG was a hydrolyzable compound whose *in vivo* TP release behavior contributed to its therapeutic efficacy. However, free TP was associated with strong toxicity toward the kidneys, liver, male reproductive system and immune system. Additionally, carbamate-induced neurotoxicity could be expected in mammals [14].

In the current study, we introduced the O-glycosidic bond in the conjugation of TP and 2-glucosamine. The glycosidic linkage was difficult to be cleaved and might stabilize the carbohydrate moiety thus making TPAG nonhydrolyzable [15]. Moreover, O-linked glycosylation represented an important modification of biologically relevant molecules such as glycoproteins, glycolipids and proteoglycans. Thus, the nonhydrolyzed triptolide derivative, triptolide aminoglycoside (TPAG), might provide an alternative in the development of renal-specific therapeutics.

Taken together, a systemic evaluation was performed of TPAG by studying the *in vitro* stability, tissue distribution, pharmacodynamic profiles and systemic toxicities of TPAG *in vivo*.

2. Experimental

2.1. Materials and animals

Triptolide (purity > 98.0%) was purchased from the Chengdu Xiyu Institute of Science (Chengdu, China). 2-glucosamine hydrochloride (99.0% pure) was obtained from Kelong Chemical Reagent Factory (Chengdu, China). Trimethylsilyl trifluoromethanesulfonate (TMSOTf) and trichloroacetonitrile (CCl_3CN) were purchased from the Chengdu Hengli Chemical Reagent Factory (Chengdu, China). All the other chemicals were commercial products of analytical or chromatographic grade. Triptolide and its derivatives were detected on TLC by spraying with the Kedde reagent or 10% H_2SO_4–C_2H_5OH. 4 Å molecular sieve was heated to 350 °C in a muffle furnace for 1 h before use. Saturated ammonia–methanol solution and highly activated zinc powders were prepared following the procedures described in the supplementary information [16]. The purification of intermediates and the final synthesized compound were performed by column chromatography on silica gel (300–400 mesh). ^{1}H NMR and ^{13}C NMR analyses were performed with an AMX-400 Bruker Spectrometer. Chemical shifts (δ units) and coupling constants (*J* values) are expressed in part per million (ppm) and hertz (Hz). HRMS was evaluated on a Bruker micro TOF-QII.

Male Sprague–Dawley (SD) rats (230–260 g) were obtained from the Experimental Animal Center of Sichuan Academy of Medical Science (Chengdu, China). Male Kunming mice (18–25 g) were purchased from the West China Experimental Animal Center of Sichuan University (Chengdu, China). All animal procedures for this study were approved by the Animal Ethical Experimentation Committee of Sichuan University according to the requirements of the National Act on the use of experimental animals (China).

2.2. Synthesis and characterization of TPAG

2.2.1. Synthesis of 2-glucosamine derivative (5)

3,4,6-Tri-O-acetyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-D-glucopyranosyl trichloroacetimidate (**5**) was synthesized as described previously [17]. The glycosidic donor was coupled to TP *via* a glycosidic bond, yielding TPAG (Fig. 1).

2.2.2. Synthesis of 3,4,6-tri-O-acetyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonyl)-D-glucopyranosyl triptolide (6)

A solution of (**5**) 765 mg (1.23 mmol), triptolide 370 mg (1.03 mmol) and 4 Å molecular sieves in dry CH_2Cl_2 (10 mL) were stirred for 30 min under the nitrogenous atmosphere and then cooled to −78 °C. TMSOTf (54 μL, 0.3 mmol) was added and the mixture was stirred for 4 h from −78 °C to 0 °C, The reaction was quenched with triethylamine, filtered and concentrated in vacuo. The residue was purified by silica gel column chromatography using toluene-EtOAc (1:1) as eluent to give white solid. The structure (**6**) was confirmed by ^{1}H NMR and ^{13}C NMR (Supplementary data Figs. S6 and S7).

2.2.3. Synthesis of 2-deoxy-2-(2,2,2-trichloroethoxycarbonyl)-D-glucopyranosyl triptolide (7)

A solution of (**6**) 850 mg (1.0 mmol) in ammonia-saturated methanol (25 mL) was stirred at room temperature for 1 h, then the reaction mixture was concentrated in vacuo. The residue which was purified by column chromatography on silica gel using CH_2Cl_2-Methanol (20:1) as eluent gave white solid (**7**). The structure (**7**) was confirmed by ^{1}H NMR and ^{13}C NMR (Supplementary data Figs. S4, S5).

2.2.4. Synthesis of TPAG (8)

To a solution of (**7**) 320 mg (0.46 mmol) in Ac_2O (10 mL) was added freshly activated Zn powder 3.0 g (46 mmol); the mixture was stirred at room temperature for 7 h. Then the reaction mixture was filtered, concentrated *in vacuo* and purified by silica gel column chromatography using CH_2Cl_2-Methanol (5:1) as eluent to give white solid (**8**) (204 mg, yield 85.1%) The structure of TPAG was confirmed by ^{1}H NMR, ^{13}C NMR and HRMS (Supplementary data Fig. S1–S3).

2.3. Sample preparation and LC–MS/MS analysis

To determine TP amount, samples for quantification were prepared as described previously [13]. For the detection of TPAG, samples (100 μL) were precipitated with 3 times (v/v) of methanol, and vortexed for 5 min, then centrifuged at 12,000 rpm for 10 min. Finally an aliquot (1 μL) of supernatant was injected into LC–MS/MS.

Liquid chromatography was performed on a rapid resolution liquid chromatography (RRLC) system (1200 series, Agilent Technologies, USA). The separation was performed on a Diamonsil ODS column (50 mm × 4.6 mm, 3 μm) with a corresponding guard column (ODS, 5 μm) with column temperature of 30 °C. The mobile phase consisted of 0.1% (v/v) aqueous formic acid and methanol (B), and the flow rate was 0.4 mL/min. For the analysis of TP, the proportion of A to B